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THE
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RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX. II.

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Analysis of the relations between metabolism and morphogenesis requires that each set of processes be separated into component parts. Metabolism may be fractionated by means of agents of which the effects on particular enzyme systems are reasonably well known and the relation of the activity of such systems to morphogenesis may then be tested. In the annelid, *Tubifex tubifex*, morphogenesis during posterior regeneration may be measured fractionally and "rate of localization," "rate of early differentiation," and "rate of later differentiation" expressed quantitatively (Collier, 1947). It was found that oxygen consumption and loss of weight by starving worms proceed at a markedly increased rate during certain stages of regeneration, and that rate of oxygen consumption was correlated with "rate of later differentiation"; a metabolic (energetic) cost of differentiation was hypothesized and it was thought possible that this might be characterized by activity of particular enzyme systems. The present report concerns the effects of continuous poisoning by cyanide and by iodoacetate and also the effects of high oxygen tension, low oxygen tension, and complete lack of oxygen upon morphogenesis during posterior regeneration in the oligochaete annelid, *Tubifex tubifex* Mull.

Materials and Methods

The worms were handled and examined as described earlier (Collier, 1947). For high oxygen tension, gas from a tank was bubbled continuously through the water in which the worms were kept. Presence of a low percentage of carbon dioxide (about 5%) was found to have no effect on experimental results. For low oxygen tension, nitrogen or hydrogen was bubbled through the water at two-day intervals, the bottles being tightly closed between treatments. For strictly anaerobic conditions, hydrogen from a tank was first freed of traces of oxygen by passing it over platinized asbestos heated to a dull red; then it was bubbled continuously through wash bottles and experimental bottles in series. That the continual disturbance did not affect regeneration was ascertained by using a control set-up through which air was bubbled.

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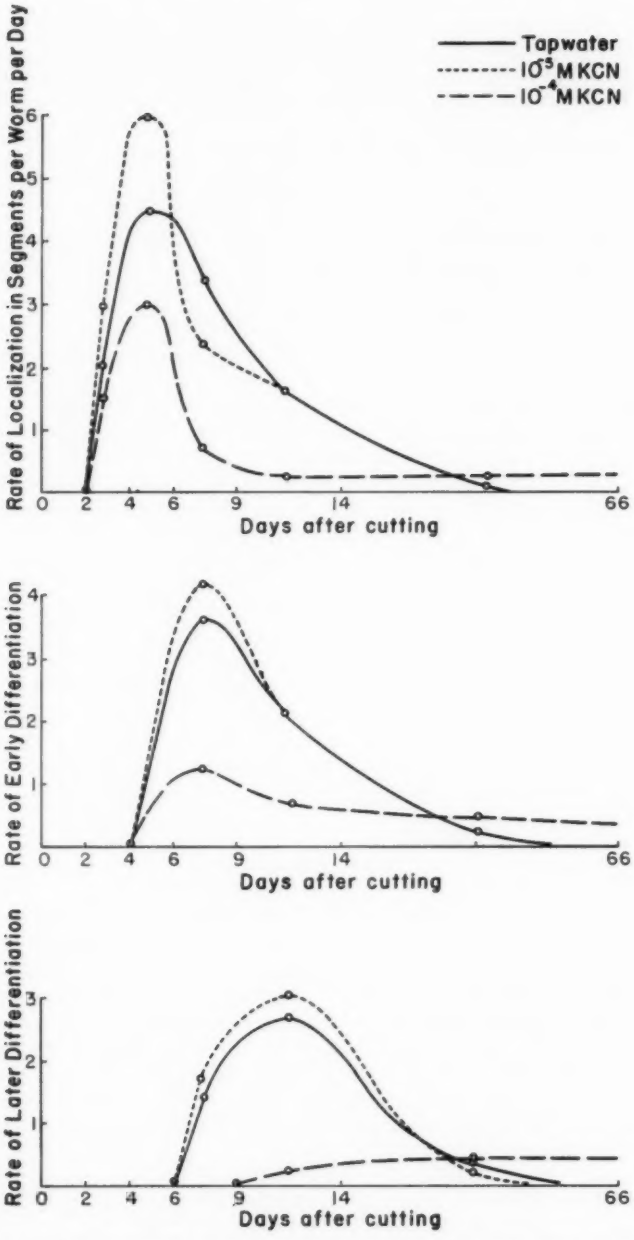

FIGURE 1. Effect of cyanide on rates of progress through various stages of regeneration. Mean deviation within each group of worms (thirty individuals) ranged from 0.3 to 0.5 segments per worm per day, increasing with time.

"Rate of localization" was calculated as increase in total number of segments per worm per day; "rate of early differentiation" as increase in number of segments showing some cellular differentiation visible *in vivo* under low power; and "rate of later differentiation" as increase in number of segments showing setae.

Effects of Cyanide on Regeneration

Over 300 worms were used in experiments involving continuous poisoning by potassium cyanide. A group of thirty worms in 10^{-3} *M* KCN became inactive and showed heavy mortality after the second day. The last survivors formed blastemae, but no localization of new segments occurred in nine days. Worms in 10^{-4} *M* KCN and 10^{-5} *M* KCN survived well, one group in 10^{-4} *M* showing 60 per cent survival at 159 days. A few individuals showed abnormal regeneration: one double tail and seven with the new tail at an angle. Since three of these eight were in a control group, the abnormalities could not be attributed to effects of cyanide.

Table I

Effect of potassium cyanide on progress of regeneration: segments per worm per day

Days	Rate of localization					Rate of early differentiation					Rate of later differentiation				
	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94
Worms in 10^{-5} *M* KCN	4.7	2.4	1.4		0.2	0	2.0	2.2		0.3	0	0	1.4		0.4
	4.8	2.4				0	2.0				0	0			
to			↘					↘					↘		
tap water			1.1		0.2			2.3		0.3			1.4		0.4
Worms in 10^{-4} *M* KCN	2.0	1.9	0.5		0.3	0	0	1.1		0.4	0	0	0		0.5
	2.4	1.7				0	0				0	0			
to			↘					↘					↘		
tap water			1.3		0.3			2.0		0.4			0		0.5
Worms in tap water	3.0	3.0	1.5	→0.8	0.2	0	2.0	2.2	→1.4	0.3	0	0	1.3	→1.9	0.4
to				↘					↘					↘	
10^{-5} *M* KCN				1.3	0.2				1.7	0.3				2.1	0.4

Data on rates of localization, early differentiation, and later differentiation are summarized in Figure 1. During the first six days the worms in 10^{-5} *M* KCN showed a significantly higher rate of localization than controls, while worms in 10^{-4} *M* KCN showed a lower rate. Later the advantage of the worms in 10^{-5} *M* KCN disappeared, but the disadvantage of those in 10^{-4} *M* KCN continued. At 66 days the worms in 10^{-5} *M* KCN and in tap-water had practically completed regeneration: all new segments were in later stages of differentiation and had grown to almost the size of the old segments. The total number of new segments was almost precisely the same for these two groups (average 41 per worm). At this time the worms in 10^{-4} *M* KCN still showed segments in the early stages of formation and the average total number of new segments was only 26 per worm. Growth of the new segments was poor. But at 159 days their condition was fully comparable with that of the others at 66 days. The total extent and perfection of regeneration were unaffected by treatment, but rate of regeneration was markedly affected.

The areas under the rate curves at the top of Figure 1 represent the average total number of segments localized per worm. For tap water and 10^{-5} *M* KCN, these areas between two days and nine days were 23 and 25, respectively, or not sig-

nificantly different, despite the early rapid rate of localization in dilute cyanide. It may be presumed that the amount of cellular material available for localization of segments was a limiting factor, that this amount was unaffected by 10^{-5} *M* KCN and that the narrowness of the peak of rate was due to more rapid exhaustion of the material. The low rate of localization in worms in 10^{-4} *M* KCN and the fact that the area under the curve from two to nine days is less than half of that under the control curve suggests that availability of cellular material may have been decreased in 10^{-4} *M* KCN. The rapid decline to a very low rate of localization strengthens this suggestion.

Since rate of early differentiation during a particular interval of time should be limited by the number of segments localized, the differences in rates of differentiation were accounted for by the previous differences in production of localized segments. It appeared probable that the cyanide affected some process or processes occurring during or preceding localization, and had no direct effect upon later processes.

TABLE II

Regeneration in low oxygen

Days after cutting	Average number of new segments per worm		
	Nitrogen	Hydrogen	Control (air)
2 to 7	blastema	blastema	blastema
10	1.4	1.5	4.7
13	2.1	2.5	7.5 (setae)
16	3.7	3.0	9.8
19	5.0	5.1	11.7
23	6.0 (setae)		13.8
26	7.0		14.7

Mean deviation was about ± 1.0 segment up to 16 days, ± 2 thereafter.

An experiment was set up to test this idea. Five groups of thirty worms each were cut for regeneration and two groups were placed in 10^{-5} *M* KCN, two in 10^{-4} *M* KCN and one in tap water. At the end of seven days one group from each cyanide solution was transferred to tap water. Also, at the end of ten days the group in tap water was separated worm for worm into two comparable groups and one of these transferred to 10^{-5} *M* KCN. Data (summarized in Table I) in general confirm the supposition that cyanide affects localization and not later processes. Further, it is suggested that this effect is not upon mobilization of neoblasts, which, according to Krecker (1923) and Stone (1932), cease their metamorphosis and migration well before ten days. Rather the effect must be upon some process more directly concerned in localization.

EFFECTS OF LOW OXYGEN TENSION

If high oxygen tension acts as a stimulus to regeneration as suggested by Barth's work on Tubularia (1940), regeneration should be inhibited or retarded by low oxygen tension, while high oxygen tension should accelerate it. Exactly opposite

TABLE III

Observations on worms kept continuously under oxygen-free atmosphere

	18 hours	30 hours	42 hours
20 intact in tap water	dark red inactive	survival about 30%	survival 15%
20 intact in 10^{-5} *M* KCN	dark red inactive	survival about 80%	survival 45%
20 regenerating in 10^{-5} *M* KCN	dark red inactive	survival about 95%	survival 25%
20 intact in 10^{-6} *M* NaIAc	dark red inactive a few dead	no survivor	
Controls with air bubbled through tap water 20 intact 20 regenerating	normal	normal	survival 100%

results would be expected from the line of reasoning that in low oxygen tension there might be an increase in glutathione (*cf.* Barron, 1951) which, as found by Coldwater (1933), can increase rate of regeneration in Tubifex. One might then expect high oxygen tension to retard or inhibit regeneration.

One hundred and fifty worms were used in two experiments with atmospheres containing less than 4 per cent oxygen (Table II). No morphological abnormalities appeared, but there was a general retardation of regenerative processes.

Results of an experiment on survival of worms under more strictly anaerobic conditions (Table III) showed that this species cannot long endure complete absence of oxygen. Survival was significantly briefer in presence of iodoacetate.

TABLE IV

Effect of lack of oxygen on early stages of regeneration. (Each day access to air was permitted long enough to allow return of normal color and activity)

	2 days	4 days	5½ days	7 days
Oxygen-free				
Tap water	survival 7/30 1/7 with blastema	survival 0		
10^{-5} *M* KCN	surv. 30/30 24/30 with bl.	30/30 with small blast.	surv. 12/30 no blastema	survival 3/12
10^{-6} *M* NaIAc	no survivor			
Under air				
Tap water	30/30 with blastema			survival 30/30 aver. 19 new segments
10^{-5} *M* KCN	15/15 with blastema	aver. 11 new segments		survival 15/15
10^{-6} *M* NaIAc	15/15 with blastema			survival 15/15

Controls gave assurance that no materials from the apparatus or wash solutions had been responsible for destruction of the experimental worms.

For the purposes of studying regeneration an experiment was set up in which survival was improved by allowing a short period of access to air once a day (Table IV). The worms did produce blastemae at the usual time but regeneration proceeded no further and the blastemae disappeared. It appears that dilute cyanide improved survival but in the absence of oxygen did not show its accelerating action on localization.

The effect of anaerobiosis on later stages of regeneration was tested using worms which had regenerated for seven days under normal conditions (Table V). The worms kept under oxygen-free atmosphere (except for twenty minutes at 27 hours) showed practically no progress in regeneration, while in the control an average of 3.6 new segments per worm had been localized, 5.7 had undergone early differentiation, and 7.1 later differentiation. Regeneration here requires the presence of oxygen.

TABLE V

Effect of lack of oxygen on later stages of regeneration

	0 hours	47 hours
15 worms under oxygen-free atmosphere		survival 100%
Aver. no. segments in localization	7.0	6.2
Aver. no. segments in early differentiation	11.2	10.0
Aver. no. segments in later differentiation	0.6	1.7
Aver. no. segments total per worm	18.8	17.9
15 worms allowed to continue under air (Control)		survival 100%
Aver. no. segments in localization	7.0	4.8
Aver. no. segments in early differentiation	11.3	9.9
Aver. no. segments in later differentiation	0.6	7.7
Aver. no. segments total per worm	18.9	22.5

Mean deviation was about ± 1.0 segment.

EFFECTS OF HIGH OXYGEN TENSION

Concurrent with the experiments with low oxygen, two groups of thirty worms each were kept under an atmosphere of 95% oxygen and 5% carbon dioxide and a third group under 100% oxygen. Results were the same in all groups. The worms survived well for several days and formed blastemae, but regeneration proceeded no further and all worms had died by ten days. To test whether inhibition of localization by oxygen and stimulation of localization by 10^{-5} *M* KCN might be based on opposite effects on the same mechanism, experiments were set up in which worms were kept in 10^{-5} *M* KCN, 10^{-4} *M* KCN, and 10^{-3} *M* KCN under an atmosphere of pure oxygen (Table VI). The 10^{-5} *M* KCN partially counteracted the effects of oxygen both upon survival and upon regeneration; 10^{-4} *M* KCN was found to partially counteract the effect of high oxygen upon regeneration, but it did not even partially counteract the lethal effect. Accordingly, high oxygen tension had an effect on regeneration independently of its lethal action and, far from stimulating regeneration in Tubifex, very high oxygen inhibits it.

EFFECTS OF IODOACETATE

Since experiments in which worms were subjected to low oxygen tension and to complete lack of oxygen had suggested that glycolysis might be important for

TABLE VI

Simultaneous effects of high oxygen tension and cyanide on survival (in fractions) and regeneration (in average number of new segments per worm)

	2 days	4 days	6 days	9 days	14 days
Under oxygen Tap water	30/30 sm. blastema	23/30 0 seg.	4/30 0 seg.	no survivor	
10^{-6} *M* KCN	30/30 sm. blastema	30/30 2 seg.	28/30 3 seg.	21/30 4 seg.	no survivor
10^{-4} *M* KCN	30/30 sm. blastema	29/30 1 seg.	no survivor		
10^{-3} *M* KCN	no survivor				
Under air Tap water	30/30 blastema	28/30 4 seg.	28/30 13 seg.	27/30 23 seg.	27/30 31 seg.
10^{-6} *M* KCN	18/18 blastema	18/18 6 seg.	18/18 18 seg.	18/18 25 seg.	18/18 33 seg.
10^{-4} *M* KCN	15/15 blastema	12/15 3 seg.	10/15 9 seg.	10/15 11 seg.	10/15 12 seg.
10^{-3} *M* KCN	29/30 no blastema	no survivor			

survival and regeneration, worms were allowed to regenerate in various concentrations of sodium iodoacetate: 10^{-3} *M*, 10^{-4} *M*, 10^{-5} *M*, 10^{-6} *M*, 10^{-7} *M*, and 10^{-8} *M* NaIAc. Five intact worms in 5×10^{-3} *M* NaIAc showed decreased activity after six hours, and were dead at thirty-six hours. In the other concentrations intact worms survived as well as but no better than the regenerating worms. The group of thirty worms in 10^{-3} *M* solution showed high mortality after three days, but the few survivors maintained normal morphogenesis. The worms in 10^{-4} *M* solution showed high mortality after six days, but six of the thirty worms survived for twenty-four days and maintained normal regeneration. Mortality in the other groups was low. Rates of progress through various stages of regeneration were very nearly the same for all concentrations of iodoacetate (Fig. 2), but whereas rate of later differentiation in tap water reached a peak between thirteen and seventeen days after cutting, the rate of later differentiation in each of the iodoacetate solutions

TABLE VII

Effect of dilute iodoacetate upon rate of oxygen consumption

Sample	Oxygen consumption in cubic millimeters per milligram of worms (wet weight) per hour	
Large worms (over 4.5 cm.)	0.12 in tap water	0.13 in 2×10^{-6} *M* NaIAc
Small worms (under 2.5 cm.)	0.14 in tap water	0.13 in 2×10^{-6} *M* NaIAc

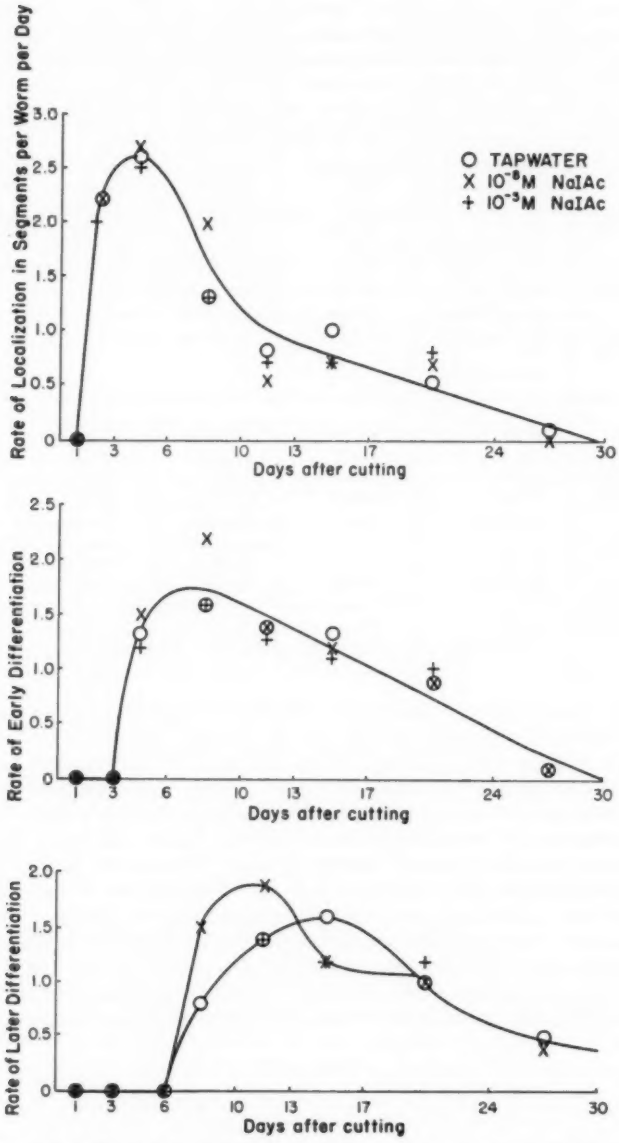

FIGURE 2. Effect of iodoacetate on rates of progress through various stages of regeneration.

reached its peak between ten and thirteen days after cutting. Iodoacetate appeared to accelerate later differentiation without affecting earlier processes. Because of the range of concentrations used, and because the extremely dilute solutions used here had maximal effect on differentiation, it may be presumed that iodoacetate poisons some process(es), inhibition of which allows further activity of some other process(es) in a system of multiple pathways of hydrogen and electron transfer (*cf.* Lipmann, 1954). The inhibited process might be glycolysis, while the reciprocally related process might or might not be concerned in the increased oxygen consumption previously found during the period of most rapid "rate of later differentiation."

A determination of the effect of sodium iodoacetate upon oxygen consumption of normal worms was made (Table VII). It is clear that iodoacetate had no significant effect on rate of oxygen consumption.

Discussion

It has been held that differences in rate of metabolism in the various parts of an animal may be the basis for production of morphological differences (Child, 1940; Hyman, 1940; Barth, 1938, 1940). Much of the supporting evidence comes from experiments on regeneration of hydroids, and the extremely rapid rate of regeneration here makes it difficult to distinguish between a factor influencing initiation of regeneration and one limiting later processes. In Tubifex, slow regeneration permits sufficient time for more detailed analysis. Since regeneration here is initiated even in the complete absence of oxygen, increased oxygen tension in the tissues at the cut surface is obviously not the primary stimulus nor is it even a necessary condition. Instead the availability of oxygen acts as a limiting factor in the progress of certain later processes in regeneration. However, the concept may be applied to morphogenesis during regeneration in Tubifex when used as Lindahl (1936) applied it in the echinoderm egg: differences in rate of particular fractions of metabolism may be the basis for certain initial processes in morphogenesis.

In the experiment in which worms with partially regenerated tails were subjected to lack of oxygen, the metabolism which supported vital processes did not support morphogenetic processes. Accordingly, regeneration must depend upon activity of some aerobic pathway. The fact that cyanide affected "rate of localization" indicates a cyanide-sensitive system important during localization. The fact that cyanide did not affect rate, extent or perfection of differentiation indicates that the particular system has little or no importance in relation to differentiation. Accordingly, on the basis of cyanide-sensitivity the processes supporting "localization" and "early differentiation" are distinct. Similarly, on the basis of sensitivity to iodoacetate the metabolic processes of "early differentiation" and of "later differentiation" are distinct.

During the period in regeneration before "later differentiation" appears, oxygen consumption was found to be only slightly, if at all, above normal (Collier, 1947). However, these worms lost weight almost twice as rapidly as controls, and this suggested an energetic cost of localization which was not reflected in oxygen consumption. The same applied to a possible cost of "early differentiation," but "later differentiation" was found associated with a markedly increased consumption of oxygen. Determination of respiratory sensitivity to cyanide showed that the increase was cyanide-stable. This contrasts with the findings of Bodine and Boell

(1934) for grasshoppers and of Sanborn and Williams (1950) for Cecropia moths, that the additional oxygen consumption during development is cyanide-sensitive although the respiration during diapause is entirely cyanide-stable. In fact it appears that the metabolic mechanisms of morphogenesis in metamorphosing insects (*cf.* Williams, 1951) can hardly be compared with those in regenerating Tubifex.

The presence of oxygen was found to be essential to "localization" but high oxygen tension inhibited it. There is no necessity for assuming that normal oxygen tension should establish optimal conditions for localization. Since these worms normally live partly submerged in mud, the optimum might be an oxygen tension lower than that established in very shallow mudless tap water under air. Fox and Taylor (1955) found this true for survival and growth of young worms in the laboratory.

Respiration as measured by the Warburg method was entirely stable to 10^{-4} *M* and 10^{-5} *M* KCN, but continuous exposure to these concentrations of cyanide affected "rate of localization." 10^{-4} *M* KCN was found to retard while 10^{-5} *M* accelerated "localization." Both concentrations counteracted the inhibitory effect of high oxygen tension.

It was considered that the accelerating effect of the more dilute cyanide solution is comparable to the often observed and seldom explained stimulation of various processes by other inhibitors in extreme dilution (*cf.* Commoner, 1940). Since in other cases the stimulation is effective upon the same processes which are inhibited by higher concentrations of the poisons, it was considered that the two concentrations of cyanide affected the same process in "localization." The concentrations of cyanide which activate proteinases *in vitro* are at least fifty times higher than 10^{-4} *M*, and were rapidly lethal to the worms (more minutely described by Hyman, 1916). Nevertheless cyanide here may have been effective upon the reactions of some metalloprotein other than those of the cytochrome system or of the haemoglobin in the blood of these worms. The antagonism of high oxygen damage by cyanide does suggest that the effects of high oxygen tension and of cyanide do meet somewhere, but if we assume that cyanide here is acting as an oxidative poison, then the cyanide-sensitive system cannot be responsible for any large proportion of the oxygen consumption: it may be off the main electron transfer pathway. The lethality of high oxygen tension also suggests an autoxidizable system that is off the main pathway (Gerschmann *et al.*, 1954). Whatever high oxygen affects, whether protein synthesis, concentration of particular normal or abnormal components, structural integrity, etc., it was at least partially counteracted in Tubifex by cyanide.

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Summary

1. Continuous exposure of regenerating *Tubifex tubifex,* Mull. to cyanide has been found to affect "rate of localization" without affecting the ultimate extent or perfection of localization or of other morphogenetic processes.

2. Continuous exposure to iodoacetate has been found to increase "rate of later differentiation" without having other effects on regeneration.

3. Low oxygen tension was found to retard regenerative processes generally. In complete absence of oxygen, blastema formation took place but all subsequent processes were effectively blocked.

4. High oxygen tension blocked morphogenesis and also was lethal in from four to eight days. Both the inhibition and the lethal effects were partially relieved by concurrent treatment with cyanide.

5. It is concluded that the availability of oxygen limits the progress of later processes in morphogenesis without playing any necessary part in the initiation of regeneration in Tubifex.

6. It is indicated that metabolic processes supporting "localization," "early differentiation," and "later differentiation" are at least partially distinct from each other and from the metabolic processes essential to maintenance; that energy released in the promotion of particular morphogenetic processes must be released through particular enzyme systems; and that such specific release of energy is essential to the progress of morphogenesis.

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THE UPTAKE OF I^{131} BY THE THYROID GLAND OF TURTLES AFTER TREATMENT WITH THIOUREA[1]

SISTER M. CLAIRE OF THE SAVIOR BILEAU[2]

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The chemical structure and relationships to goitrogenicity of several hundred compounds have been tested by a number of investigators, for example: the Mackenzies (1943); Astwood, Sullivan, Bissell and Tyslowitz (1943); Astwood (1943); Taurog, Chaikoff and Franklin (1945); Astwood, Bissell and Hughes (1945); McGinty and Bywater (1945) and VanderLaan and Bissell (1946). In general the active substances fall into one of three classes: 1) thiourea and its derivatives, 2) aniline derivatives, including the sulfonamides and other aminobenzene compounds, 3) thiocyanates and organic cyanides. The most active compounds tested possess a thiourea grouping or thioureylene radical —NH·CS·NH—. Replacement of the hydrogens of thiourea by methyl groups increases its activity, a fact which suggests the importance of the thio rather than the mercapto grouping for the activity of this class of substances. Extensive reviews concerning antithyroid agents have been published by Charipper and Gordon (1947), Astwood (1949) and Comsa (1953).

Vertebrates of every class have been tested for their reaction to antithyroid drugs. Such studies, however, for the most part, have dealt with mammals. Gordon, Goldsmith and Charipper (1943) made the first report of the use of inhibitors on the thyroid gland of cold-blooded animals. Since then a number of investigators have studied the effects of goitrogenic substances on poikilotherms. Lynn and Wachowski (1951) have published a comprehensive review of the literature dealing with the thyroid gland and its functions in cold-blooded vertebrates.

Little work has been done concerning the function of the thyroid in reptiles. Ratzersdorfer, Gordon and Charipper (1949), Adams and Craig (1951) and Fisher (1953) have reported the effects of antithyroid compounds on the lizards. Naccarati (1922) described the normal histology and gross anatomy of the thyroid gland of the turtle *Emys europa,* while Evans and Hegre (1940) studied seasonal changes and the effects of pituitary extract on the thyroid of *Chrysemys picta belli.* Greenberg (1948) was the first to investigate the effects of thiourea on the histology of the thyroid gland of the turtle. In her work immature specimens of *Pseudemys elegans* were used. Since then, Adams and Craig (1950) and Paynter (1953) have studied the thyroidal response to goitrogens in *Chrysemys picta picta.* Pastore

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(1950) investigated the effects of thyroid-stimulating and thyroid-inhibiting drugs upon the histology of the thyroid of *Clemmys insculpta* and *Graptemys geographica*. Dimond (1954) studied the reactions of developing snapping turtles, *Chelydra serpentina serpentina*, to thiourea.

Investigators who have worked on the thyroid gland of turtles are unanimous in pointing out, as an explanation for the observed irregular reactions to goitrogenic agents, what appears to be an inherent variability. This variability far exceeds that found in warm-blooded vertebrates. Uhlenhuth, Schenthal, Thompson, Mech and Algire (1945) working with the newt, *Triturus torosus*, claimed that such a high degree of variation does not seem explicable on the basis of the known physiological roles of the urodele thyroid and must be attributed to what might be called a general instability of the endocrines in cold-blooded vertebrates.

Some of the methods used to acquire a more thorough knowledge of the gland are: 1) gravimetric methods, based on changes in thyroid weight; 2) chemical methods, a quantitative as well as a qualitative study of iodine in the gland; 3) the use of radioisotope technique (radioactive iodine in the thyroid increases progressively with increasing dosage of TSH); 4) histological methods, which study mainly: a) epithelial height, b) staining reactions of colloid and cells, c) position and shape of nuclei and nuclear volume; 5) microhistometric methods. Uotila and Kannas (1952) have devised a linear measurement method, which permits quantitative determinations of the principal components of the thyroid tissue. The method appears to have the advantage of objectivity, simplicity and economy of time. This method was compared with the planimetric method and cell height measurement by Tala (1952) and found to give an accurate picture of the histological activity of the thyroid gland.

The use of radioactive iodine in experimental studies on the thyroid gland has added considerably to present-day understanding of the histophysiology of this organ. The early studies of Hertz, Roberts, Means and Evans (1940), Hertz and Roberts (1941), and Hamilton and Soley (1940), in which radioactive iodine was used for the first time in thyroid investigation, ushered in a technique which enabled more precise interpretation of the relationship between iodine metabolism, the thyroid and the hypophysis.

Tracer studies, using radioiodine after treatment with thyroid-inhibiting and thyroid-stimulating substances, are perhaps the most widely applied tools in thyroid investigation today. These recent developments are being used extensively in studying the thyroid function in mammals and are now being employed to some extent on cold-blooded vertebrates. However, as stated above, very few experiments have been performed on the reptiles. This is a particularly significant gap in our knowledge, in view of the fact that reptiles are considered as the stem of the birds and mammals in the vertebrate scale, and as the only cold-blooded amniote. One of the main reasons for this neglect has been the difficulty in performing thyroidectomy in these animals. The recent development of an effective "chemical thyroidectomy" opens new opportunities for research in this field. Already, some attempts have been made at correlating cell height, dry weight of the thyroid and the histological picture with the uptake of radioiodine in cold-blooded vertebrates such as salamanders (*Desmognathus fuscus* (Rafinesque); Fisher, 1953) and in the turtle *Chrysemys picta picta* by Paynter (1953). The results obtained in these experiments point again to the greatest difficulty encountered in the study of thyroid

function at this level, namely, the astonishing variability. The present work is an attempt at investigating some of the factors which may influence this great variability in the function of the thyroid of the turtle *Chrysemys picta*. To this effect a study was made of the possible correlations between radioiodine uptake and colloid level, percentage epithelium, cell height and dry weight of the thyroid, in normal and in treated animals; an endeavor was also made to determine the thyroid/serum ratio, when organic binding is blocked and when binding is permitted.

Materials and Methods

Turtles with carapace length between five and seven inches were purchased from The Lemberger Company, Oshkosh, Wisconsin and the J. R. Schettle Frog Farm, Stillwater, Minnesota.

The experimental and control animals, totaling one hundred sixteen, were kept in large metal tanks which were arranged so that the animals had free access to running water or dry perches. The temperature of the room in which the tanks were located was kept as close to 75° F. as possible. All animals used in this series of experiments were denied food during the term of treatment since the amount of iodine in the food could not have been controlled.

The experimental animals were given subcutaneous injections of thiourea three times a week. The dosage, found most effective by Paynter (1953) for these experiments, was 0.25 cc./100 gr. of body weight of a 0.1% solution. After four, six and eight weeks of treatment the animals were injected with a tracer dose of three microcuries of carrier-free I^{131}, in 0.5 cc. of distilled water. The untreated control animals received the same dose of I^{131}. Three hours after administration of the radioactive iodine the animals were sacrificed. The plastron of each turtle was removed, blood was taken from one of the large vessels leaving the heart and allowed to clot. Immediately after exsanguination, the thyroid gland was removed and cut in half. The right half was cooled by placing it on dry ice. The left half, in 27 of the experimentals and 20 of the control animals, was immersed in isopentane, cooled by liquid nitrogen. Afterward, the glands, placed in individual tubes, on the surface of degassed paraffin, were transferred to the drying chamber of a freezing-drying apparatus (Altmann-Gersh technique; Gersh, 1932). Dehydration was carried out at − 40° C. and continued for a minimum of 72 hours. The system was then gradually brought to the melting point of paraffin by immersing the drying chamber in a beaker of water which was kept at constant temperature by placing it on a thermostatically controlled hot plate. The vacuum was broken only after infiltration was completed.

As for the remainder, 42 experimental and 27 control animals, the left half of the gland was placed on a glass slide and dried at 37° C. to determine dry weight. These glands were recovered by soaking in 0.025% solution of trisodium phosphate for 24 hours and in 10% formalin for three hours.

The right half of the gland, for each animal, was placed into glass homogenizers containing 6 ml. of ice-cold distilled water and one mg. of NaI as carrier, and homogenized immediately. The homogenate was deproteinized according to Somogyi (1945). To 0.80 cc. of homogenate, 0.10 cc. each of $Ba(OH)_2$ and $ZnSO_4$ were added, followed by vigorous shaking, and centrifuging. The supernate was removed with a pipette, three drops were placed on each of three glass sides. The

same was done with the precipitate and a sample of the homogenate before deproteinization. These were dried at 37° C.

The serum was deproteinized by diluting a 0.05-ml. sample to 0.08 ml. with water containing 0.10% NaI and adding to it 0.10 ml. each of $Ba(OH)_2$ and $ZnSO_4$ as above. To determine the I^{131} content of the serum an aliquot of diluted serum (0.05 ml. diluted to 1.00 ml. with water containing 0.10% NaI) was dried on a glass slide.

Precipitable and non-precipitable I^{131}, as well as total I^{131} in both gland and serum, were measured with a Geiger-Mueller tube (window thickness 1.7 mg./cm.$^{-2}$) placed at a distance of 4 mm. from the object. The counts were also determined for the glands of both series, frozen-dried and oven-dried. Three determinations of one minute duration each were taken and averaged. The counts were brought up to the value on the day of injection by the use of decay factors which were based on the eight day half-life of I^{131}.

The glands were sectioned at 5 micra and those of the frozen-dried series were fixed by floating on 10% formalin and amphibian Ringer's solution and stained with Gomori's chrome alum hematoxylin and phloxin. Three sections were selected at 25%, 50% and 75% of each gland. Film strips of these sections were made and projected on white paper which served as a screen, using a magnification of × 100. Two lines, intersecting at obtuse angles in the form of an ×, were drawn in advance on the plane to which the image was to be projected. The image of the stained specimen was positioned so that its center fell approximately on the junction of the intersected lines. The outline of the follicles and colloid along the full length of the two lines was drawn. The segment of the lines covered by the entire figure was then measured in millimeters. Similarly, millimetric lengths of the segments covered by epithelium and colloid were determined. The total of the epithelium segments, divided by the whole length of the lines, gave the percentage of epithelium. The percentage of colloid was calculated in the same way. This linear measurement method of determining the principal components of the thyroid gland was devised by Uotila and Kannas (1952) and further tested by Tala (1952).

Observations

It is fairly well established from animal experimentation that antithyroid compounds of the thioureylene type owe their activity to their property of preventing the organic binding of iodine, or, what amounts to the same thing, the inhibition of the oxidation of iodide to iodine. Under the influence of these agents, iodide is still able to concentrate in the thyroid gland, but it remains in a reduced, ionic state. As a result of treatment with thiourea it should then be expected that radioiodine uptake would be higher in the experimental animals than in the corresponding control groups. This fact was well brought out in the course of the study; in all cases the counts were much higher in the treated animals than they were in the untreated. Analysis of the results based solely on length of treatment were much too variable to draw significant conclusions. Re-interpretation of these results on the basis of seasonal cycles gave a more valid picture.

A total of 72 animals were treated with thiourea; 52 of them received 18 subcutaneous injections over a period of six weeks and 20 received 12 injections over a period of four weeks. Forty-seven animals were used as controls and received

three microcuries of radioiodine at the same time as the treated animals, three hours before they were killed. One set of controls was examined each time an experimental group was run.

An analysis of the results from two different points of view, 1) length of treatment and 2) seasonal factor, follows.

Length of treatment

From this point of view, the outstanding feature throughout the entire study is the great variability observed in control groups as well as in experimental animals. Taking the various correlations specifically the following can be reported.

Six-week series. The percentage epithelium was lower and the range of variation in percentage epithelium was considerably higher in experimental animals. The radioiodine uptake per unit epithelium and the range of variation in uptake were both higher in the treated than in the control group. The coefficient of correlation between the per cent epithelium and radioiodine uptake was -0.38 for the experimental group and -0.31 in the control animals. It becomes evident from the above figures that the correlations between per cent epithelium and uptake were practically nil in both treated and untreated groups.

The percentage colloid, radioiodine uptake per unit per cent colloid, and the range of variation were considerably higher in the treated animals than in the control group. The coefficient of correlation for the colloid and uptake was -0.19 in the treated animals, while it was -0.69 in the control group. In terms of colloid uptake the correlation was slightly improved in the treated animals but remained insignificant in the control animals.

Four-week series. There was no significant difference between the epithelium percentage and colloid level of experimental and control animals. The radioiodine uptake was much lower than in the six-week group and the range of variation was greatly reduced. The coefficients of correlation were as follows: per cent epithelium and uptake, control -0.20, experimental, -0.06; unit colloid and uptake, control -0.10, and experimental -0.27. The correlations in the four-week series were considerably improved but even if the range of variation was reduced it was still too high to permit significant correlations.

From the point of view of length of treatment it was impossible to establish significant correlations, due to the high range of variation. The correlations were poorest in the six-week series, but became somewhat improved in the four-week series. This was particularly true in regard to colloid level and iodine uptake.

Seasonal factor

During the course of this study the influence of a seasonal factor has been observed. Since the animals had been obtained from the supplier at three different times of the year, the three series therefore varied as to the time of the year during which the experimental work was done. When the results were analyzed in terms of seasonal cyclic activity, the correlation between epithelium or colloid and uptake was greatly improved and the variability was decidedly reduced, especially in the fall series. The first series was carried out during June and the beginning of July. The second series was carried out during November and the last series during the month of February and the beginning of March. As a result of such spacing, the

TABLE I

Radioiodine uptake per milligram dry weight. Difference in per cent of controls

Series	Mean count per milligram		% of control uptake
	Experimental	Control	
Winter	592.29	527.01	112.38%
Summer	985.34	534.85	184.22%
Fall	484.16	378.55	127.89%

possibility of a seasonal factor controlling the activity of the thyroid gland in the turtle was brought out, and this factor appeared to exercise its influence even in the treated animals.

Uptake per milligram dry weight. In the determination of radioiodine uptake, it was observed that 1) the counts per minute per milligram dry weight were higher in the treated animals than in the corresponding control animals, and 2) the counts for both experimental and control animals were highest during the summer and lowest in the fall series. This last point is a strong indication of the influence of a seasonal factor and is in agreement with Eggert (1935) who reported highest thyroid activity in June, in the case of hibernating lizards, and lowest activity during December and January. In this case the winter group, having been killed at the end of February and the beginning of March, would correspond to the resumption of the secretory activity. A summary of the results in per cent of control is given in Table I.

Thyroid/serum ratio. Measurements of the thyroid/serum ratios were made when organic binding was blocked with thiourea and when binding was permitted. Due to the high degree of individual variation, the time required for the maximum uptake, as determined by Paynter (1953), was found to vary considerably with

TABLE II

Per cent epithelium. Experiments in the order of their ranges of variation

Series	Seasons	No. of cases	Mean % epithelium	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	15.18	18.33–11.44	6.89	45.38%
	Summer 6 weeks	11	16.18	20.90–10.27	10.63	65.69%
	Fall 4 weeks	18	16.22	18.18–14.26	3.92	24.16%
Control	Winter	9	17.01	20.09–12.86	7.23	42.50%
	Fall	13	16.77	19.84–12.70	7.14	42.57%

each animal. Consequently a significant ratio could not be established. The effects of the seasonal cycle, however, could still be observed. In taking the counts per minute for the homogenate and comparing it with the counts for the serum the following results were obtained:

1) The summer group: in 92% of the experimental and 40% of the control animals, the thyroid homogenate had a higher count than the serum.

2) The fall group: in 43% of the experimental and 20% of the control animals, the thyroid homogenate had a higher count than the serum.

3) The winter group: in 36% of the experimental and 16% of the control animals, the thyroid homogenate had a higher count than the serum.

The results bring out very clearly the influence of a seasonal cyclic activity. The values obtained for the summer groups are almost three times as high as those for the winter groups and twice as high as the values obtained for the fall series. These differences appear to be directly correlated to the phase of activity of the thyroid gland in hibernating reptiles.

TABLE III

Colloid level. Difference between ranges of controls and experimentals

Season	Series	Colloid level Maximum-minimum	Range	Difference between experimental and control ranges	
				In points	In % of control range
Winter	Control	75.50–63.12	12.38		
	Experimental	78.93–54.86	24.07	+11.69	+94%
Fall	Control	80.87–67.59	13.30		
	Experimental	78.74–60.67	18.07	+ 4.77	+35%

Per cent epithelium and iodine uptake. Two six-week series were carried out, one during late February and early March and the other during June and July, and one four-week series during November. The range of variation was too high to establish significant correlations between the per cent epithelium and radioiodine uptake, but the influence of the seasonal factor was nevertheless observed. The per cent epithelium in the treated animals was higher in the summer, with no significant difference in percentage for the fall group, but decidedly lower for the winter. In the control series, comparisons between percentage epithelium or colloid level are available only between the fall and winter groups. The summer control animals were not used for measurements. The range of variation in the three series gave a definite indication of a seasonal cyclic influence. Table II lists the per cent epithelium with the range of variation for the three seasons during which the work was carried out.

The uptake of radioiodine per unit per cent epithelium was considerably higher during the summer and lowest during the fall. Here again the results seem to indicate that the state of activity of the thyroid gland in the painted turtle corresponds

TABLE IV

Radioiodine uptake per unit colloid. Difference between ranges of controls and experimentals

Season	Series	Radioactivity Maximum–minimum	Range	Difference between experimental and control ranges	
				In points	In % of control range
Winter	Control	122.74–10.88	111.86		
	Experimental	238.24– 6.58	231.86	+119.82	+107%
Fall	Control	197.51– 4.59	192.92		
	Experimental	129.76– 6.58	123.18	– 69.74	– 36%

rather closely to the seasonal cycle as described above and in agreement with the work of Eggert (1935).

The counts per unit epithelium in the treated animals were 383.63 for the summer, 335.79 for the winter and only 157.13 for the fall groups. Comparing the counts for the untreated animals the same general cyclic activity could be observed. The correlations, even though somewhat improved, still remained insignificant because of the range of variation.

Colloid level. The influence of the seasonal factor became more evident in the studies on correlation between colloid level and radioiodine uptake. The cyclic pattern was decidedly in accordance with the various phases of activity described for the hibernating reptiles. The mean uptake per unit colloid level was 79.91 for the summer, 65.31 for the winter, and 33.86 for the fall group in the treated animals.

The best evidence for this factor was brought out in the closer correlation be-

TABLE V

Colloid level experiments in the order of their ranges of variation

Series	Seasons	No. of cases	Mean % colloid	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	69.46	78.93–54.86	24.07	34.65%
	Summer 6 weeks	11	75.95	87.01–60.54	26.47	34.86%
	Fall 4 weeks	18	72.76	78.74–60.67	18.07	24.83%
Control	Winter	9	69.24	75.50–63.12	12.38	17.87%
	Fall	13	71.81	80.87–67.59	13.30	18.52%

tween the colloid level and the uptake of radioiodine in the fall group. The range of variation is at its lowest in both colloid level and range of uptake. Tables III and IV give the difference between the ranges of variation for colloid level and uptake in per cent of control range. It is to be observed that for the fall group the variation in colloid level for the experimental group was 35% above the control, as compared with 94% for the winter series. The range of variation of iodine uptake in experimental animals for the fall series was 36% below the controls and 107% above in the winter series.

From the above observations it was possible to conclude that in terms of length of treatment, the range of variation in uptake of radioiodine, either per unit of epithelium or colloid level, was much too high to establish significant correlations. When, however, the results were analyzed from the point of view of seasonal cyclic activity it became apparent that the gland was under the influence of a seasonal factor which exercised its control in treated as well as in untreated animals. More

TABLE VI

Radioactivity/unit % colloid. Experiments in order of time of treatment

Series	Seasons	No. of cases	Mean radioactivity per unit % colloid	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	65.31	238.24– 6.58	231.68	354.74%
	Summer 6 weeks	11	79.91	141.83–18.69	123.14	154.09%
	Fall 4 weeks	18	33.86	129.76– 6.58	123.18	363.79%
Control	Winter	9	57.99	122.74–10.88	111.86	192.89%
	Fall	13	40.61	197.51– 4.59	192.92	475.05%

complete data on colloid level and uptake and variation are given in Tables V and VI.

DISCUSSION

Antithyroid compounds may inhibit the normal function of the thyroid gland by acting directly on the thyroid itself at any one of the stages of hormone production: 1) the collection of iodide from the circulation; 2) the synthesis of thyroid hormone; 3) the release of hormone to the tissues.

Thiocyanate ions exert a unique effect upon the thyroid gland shared by no other substance yet known (Astwood, 1949). Animals treated with this substance have been shown to be unable to collect iodide from the circulation. Other substances, like thiourea and thiouracil, are believed to block an enzyme system, thereby preventing organic synthesis of the hormone, but having no effect on the "iodide trap."

Astwood and Bissell (1944) found that in rats under the continuous influence of thiouracil the iodine content of the thyroid rapidly falls to low levels and that the thyroid gland simultaneously enlarges. Astwood (1944–45) showed that animals thus depleted of iodine are still able to concentrate rapidly considerable quantities of iodine when injected with potassium iodide.

D'Angelo, Paschkis, Cantarow, Siegel and Riviero-Fontan (1951) have observed that despite uniformly decreased radioiodine uptake with chronic propylthiouracil treatment the total radioactivity in the thyroid eventually exceeds normal when sufficient hyperplasia has occurred to offset the limited uptake. The augmented radioiodine collections which result where the drug is withdrawn, however, are greater than would be expected from hyperplasia alone, and must result in part from increased avidity of thyroid tissue for the radioactive iodine. The augmented avidity for iodine upon withdrawal of the drug is demonstrable after periods of treatment too short to have caused hyperplasia, although it increases progressively with longer periods of treatment and consequently with thyroid hyperplasia before the goitrogen is withdrawn.

The results obtained in this study are in agreement with the above observations. The uptake of radioiodine was considerably higher in the animals in which binding was blocked as compared with those in which binding was permitted. This rapid uptake of iodine by the thyroid gland, reaching a maximum and followed by a discharge of the trapped iodine, varies with the type of animals used. In mammals, for example, the time required for maximum concentration may be as little as ten to fifteen minutes as reported by Hertz, Roberts, Means and Evans (1940) and Chaikoff and Taurog (1949).

In cold-blooded animals, however, great variations are observed in the reactions of the thyroid, and because of this great range of variability the maximum uptake in the turtle would seem to be controlled by a factor other than time alone.

In accordance with Adams and Craig (1950), Paynter (1953) and Dimond (1954) the results obtained in this investigation show that the reactions to thiourea in the turtles are not as profound as those reported in warm-blooded animals, and especially in mammals. The percentage epithelium was slightly lower in treated animals than in control animals, while the colloid was higher in the experimental than in the control animals. This could be explained by the fact that the experimental animals were injected with a tracer dose of I^{131} 24 hours after the last treatment with thiourea. This delay would allow the pituitary-thyroid axis to be restored to normal conditions, and consequently after a period of increased activity, the thyroid had stored a considerable amount of colloid.

Seasonal factor. A discussion of the seasonal physiology of any vertebrate immediately arouses an inquiry concerning the behavior of its endocrine glands, especially that of the thyroid and the pituitary because of the reactions to temperature by the former and the close association and control of the thyroid function by the latter.

In general, throughout the vertebrate classes, it may be said that if a species is inactive (hibernating) during the winter months, the thyroid is inactive at this time and will not become active until the animal resumes activity. Warm-blooded animals for example, which are active all winter, have thyroids reported to be more active during the cold season, in order to maintain their normal body temperature and BMR. The same animals have lower rate of thyroid activity during the sum-

mer, the temperature of the environment tending to prevent the loss of body temperature, thereby lowering the energy requirement for the maintenance of a normal BMR.

It can generally be said that the reverse is true in cold-blooded animals. However, the seasonal conditions of thyroids in cold-blooded vertebrate hibernators are less well known. Morgan and Fales (1942) reported that there are comparatively few observations on the full seasonal cycles of the thyroid of amphibians and some of these are conflicting. Several observers are in agreement concerning the seasonal condition of the thyroids in various species of frogs and toads, all of which are hibernators. In the main they report a winter phase of moderate activity and a summer phase of greatly lowered activity. Burger (1946), in his observations on seasonal conditions of the thyroid of the male of three species of urodeles, reports variations between animals and between individual follicles of the same gland. The three species, however, all showed a similar broad cyclic pattern in that activity was highest in the spring, lowest during the summer and moderate in the fall. Similar results have been reported by Miller and Robbins (1955) for the urodele amphibian *Taricha torosa* (*Triturus torosus*).

Although few in number, some studies of the seasonal cycle of the thyroid of reptiles have been made on hibernating and non-hibernating species. Eggert (1935) studied three forms of European Lacertas and reported hibernation beginning from the end of September reaching a peak in December and January when the gland is at its most reduced activity. Young animals resume their secretory activity in February and attain their highest activity in June. Seasonal variations in the thyroid of lizards have also been noted by Ratzersdorfer, Gordon and Charipper (1949) in *Anolis carolinensis*. In non-hibernating *Xantusis vigilis*, Miller (1955) has observed that the cycle is closely correlated with the various phases of the life history of the animal. He reports the lowest thyroid activity for the fall and an increase in activity during the winter. This increase in activity during the winter may be related to the fact that the animals are active and feeding during the coldest months of the year. The influence of the seasonal factor has also been observed in the cyclic activity of the thyroid in turtles. Evans and Hegre (1940), working with *Chrysemys*, obtained results which resembled those of typical hibernating forms even when the animals were fed at regular intervals and kept at room temperature (70° F.) throughout the fall and winter. This they claim would indicate that the thyroid gland of the turtle was under the control of a genetic factor. This factor exercises its influence independently of the temperature of the environment.

During the course of this study the influence of a seasonal factor has been observed which is in accordance with the results obtained by Uhlenhuth, Schenthal, Thompson and Zwilling (1945); Uhlenhuth, Schenthal, Thompson, Mech and Algire (1945); Evans and Hegre (1940) and Greenberg (1948). The experimental animals had been treated with a 0.1% solution of thiourea, a concentration claimed to be most effective by Paynter (1953). It would seem, however, that since turtles and reptiles in general appear to be more refractory to thiourea, a higher dose would produce more marked results. Despite the weak responses and the great range of individual variation, the results were more significant when interpreted in terms of seasonal activity.

The uptake of radioiodine by the thyroid gland was always considerably higher for the summer groups, treated and untreated, whether it was considered from the

point of view of uptake per milligram dry weight; uptake per unit epithelium, unit colloid or thyroid/serum ratio. The uptake for the fall and winter series was much lower than that for the summer series. This is in agreement with Eggert (1935) who reports highest thyroid activity in June, in the case of hibernating lizards. The November series then would be nearing the most reduced thyroid activity which in lizards occurs during December and January. The winter group, killed at the end of February and the beginning of March, would then be expected to show resumption of secretory activity. The thyroid gland of the untreated control animals was observed to have a lower uptake of radioiodine per unit; however, the results were shown to follow the same general pattern as the treated animals. These results gave further evidence in favor of the seasonal cyclic activity.

The best correlations were found to occur in the fall series. The epithelium percentages of experimental and control animals were more closely related than in the other series; the range of variation in per cent epithelium was lower in the experimental animals; the correlation between epithelium and radioiodine uptake was definitely improved. The seasonal influence, however, was best demonstrated by the better degree of correlation between the colloid level and the radioiodine uptake. It was observed that the colloid level was lower in untreated controls than in treated thyroids. This may be due to the avidity of the treated thyroids for the iodine, since the injections of I^{131} were given 24 hours after the last treatment with thiourea. Considering the fact that the colloid is known to decrease during the active phase of the thyroid gland, it is not surprising that a lower colloid level was observed in the thyroids of controls for the February–March series than in the November series, which was during the phase of decreasing activity and colloid storage.

Since the turtles were kept in a room where a fairly constant temperature was maintained throughout the experiments, it would seem that the seasonal factor which regulates the cyclic activity of the thyroid in turtles is independent of the environmental temperature and probably is genetic in nature.

Summary

1. The painted turtle, *Chrysemys picta* (Schneider), was treated with a 0.1 per cent solution of thiourea by means of subcutaneous injections, then injected with radioiodine in order to determine the correlation of percentage epithelium and colloid level with radioiodine uptake. The data obtained in this study were analyzed statistically in terms of length of treatment and the time of the year during which the work was carried out.

2. From the point of view of length of treatment, the correlations between the uptake of I^{131} and colloid level or per cent epithelium were very poor, due to the high degree of variability.

3. When these results were analyzed from the point of view of seasonal cyclic activity, the correlations were decidedly improved.

4. The best correlations were obtained in the fall group, where the percentage epithelium of experimental and control animals was more closely related; the range of variation was decidedly lower, and the correlation between colloid level and uptake of radioiodine much better than in the other series.

5. Further evidence in favor of the seasonal cyclic activity was found in the uptake per milligram dry weight, and in the thyroid/serum ratio.

6. This seasonal factor appears to be genetic in nature since it exerts its control independently of the environmental temperature and the effects of the drug.

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SOME FACTORS CONTROLLING THE INGESTION OF CARBOHYDRATES BY THE BLOWFLY [1]

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Diet selection and preference are commonly evaluated in terms of quantity of food consumed; however, measurements of intake alone give little information concerning the degree to which different factors participate in the regulation of ingestion. It is clear in the case of insects that a sequential contribution by various stimuli governs the finding of food, the initiation of biting or sampling, the continuance of feeding, and the termination of feeding. It is believed by some (*e.g.*, Dethier, 1953; Fraenkel, 1953) that stimuli which initiate sampling and which drive continued feeding are neither necessarily nor invariably correlated with nutritional values. Other workers, notably Kennedy (1953), believe that there is an important causal relationship between stimulating and nutritional characteristics. The present study is intended as a step toward the ultimate clarification of this problem.

Carbohydrates were chosen as test compounds because they do not stimulate the olfactory sense and because they represent all possible combinations of stimulating effectiveness and nutritional value. There are sugars which are stimulating but non-nutritional, stimulating and nutritional, non-stimulating but nutritional, and non-stimulating and non-nutritional. Sugars representing these categories were employed in the following experiments: (1) preference-aversion tests in which were recorded the volumes imbibed by flies given a choice between sugar and water or between one sugar and another; (2) individual feeding tests in which volume intake was measured in the absence of a choice situation; (3) tests of the sensitivity of the different chemoreceptor systems to stimulation; (4) measurements of the volume intake of mixed solutions; (5) longevity tests to ascertain the nutritional value of the various sugars at different concentration levels.

Materials and Methods

Preference-aversion tests were conducted according to the procedure of Dethier and Rhoades (1954). In essence, the tests consisted of presenting groups of twenty flies, which had been enclosed in one-quart mason jars, with the choice of drinking from either or both of two J-shaped volumetric pipettes. The mean per capita fluid intake per twenty-four hours was calculated from the total volume of fluid taken from each pipette over a four-day period. In two-choice situations of this sort the intake of sugar can be compared with that of water or of any other sugar or sugar mixture.

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In order to ascertain the number of visits which were made to each pipette and the duration of each visit, the original apparatus was modified as follows. A silver wire was inserted into each pipette in such a way as to extend the entire distance from the large opening to a point just one millimeter short of the capillary orifice. Silver-conducting paint (DuPont Silver 4916) was then brushed in a thin line from a point near the large orifice to a point one millimeter short of the capillary orifice; here the painted line was extended around the circumference of the pipette so that a fly had to stand on the paint in order to drink. To the painted line near the large opening was soldered a silver wire. This wire and the wire from inside the pipette were each extended to the terminals of a Brush BL907 amplifier which in turn was connected to a BL202 recording instrument. Since the entire apparatus was intentionally unshielded, the two wires acted as antennae which picked up 60 cycle current from lights and various motors operating in the laboratory. Whenever a fly attempted to drink from a pipette, it closed the circuit between the conducting

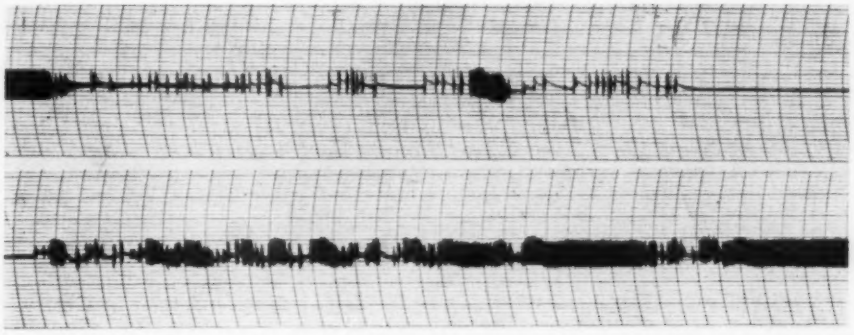

FIGURE 1. Typical example of automatically recorded periods of feeding. The thin line represents periods of feeding. Note that the fly has taken one long drink beginning at the upper right and continuing at the lower left. During the remainder of the time only brief samples were taken. Each curved line represents 5 seconds.

paint on which it was standing and the fluid and wire within the pipette. Since the 60 cycle current was then shorted out, the period of drinking appeared on the record as a straight line instead of alternating impulses (Fig. 1). The authenticity of records obtained by this method was confirmed by visual observation. At the same time the identity of the drinker was noted.

Finally, the fluid intake of individual flies was measured by direct analyses of sugar. For these measurements the flies were fed 24 ± 2 hours before testing on 0.1 *M* sucrose and then received neither food nor water until the experimental ingestion. At this time the flies were mounted on waxed sticks and individually fed on the test solutions. Some arbitrary criterion of repletion was necessary since a fly will continue alternately to extend and retract its proboscis almost indefinitely on some sugars, all the while taking small additional amounts. Repletion, therefore, was defined by the period of vigorous proboscis extension and active uptake. Usually a fly would feed continuously and actively for an initial prolonged period and then perhaps for an additional shorter period when its labellar hairs were brought

into contact with the solution. This period of active feeding was usually rather sharply delineated, as indicated by the agreement of duplicate determinations on different groups of flies treated similarly. The standard deviation of replicate determinations of volume intake ranged between 0.377 μl for 1 M sucrose and 1.34 μl for 1 M fucose.

The determination of quantity ingested was accomplished by a sensitive spectrophotometric reaction for carbohydrates employing anthrone in concentrated sulfuric acid (Dimler *et al.*, 1952). For each determination the abdomens of 5–20 flies were ground, immediately after feeding, in 10 ml. of 5% trichloroacetic acid. The crop and intestine, which contain the ingested sugar, are located entirely in the abdomen after feeding. Equally large groups of flies similarly treated, but fed nothing, served as controls. After centrifugation, aliquots of the supernatant were diluted appropriately to produce concentrations from 30 to 200 μg. sugar per ml.

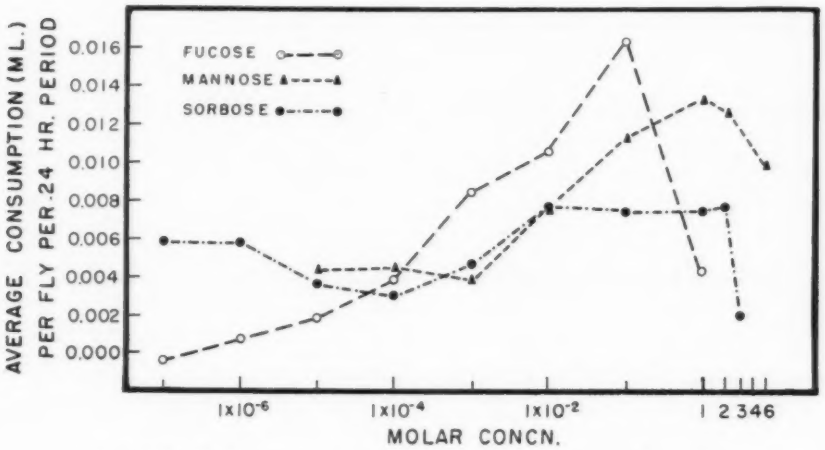

FIGURE 2. Preference-aversion curves for fucose, sorbose, and mannose.

One-ml. samples of these final dilutions were employed for the anthrone reaction and were compared spectrophotometrically with standards of the several sugars fed. The amount of sugar in the fed groups in excess of that in the unfed controls was directly convertible to volume since the concentrations of the solutions employed for feeding were known precisely. Longevity was measured with the same experimental set-up employed for preference-aversion testing.

PREFERENCE-AVERSION CURVES

The volume intake, as compared with water, was measured for each of the following sugars over the concentration range 1×10^{-7} M to saturation: fucose, sorbose, mannose. The results are summarized in Table I and Figure 2.

Fucose is a methyl pentose which is rather effectively stimulating for the tarsal chemoreceptors (median acceptance threshold = 0.087 M) but not utilized by the

TABLE I

Amount of solution consumed (ml./fly/24 hrs.) when sugar is paired with water

Molar concen.	Fucose	Water	Level (%) of significance of difference
1.0	0.0042	0.0035	—
0.1	0.0161	0.0012	0.1
0.01	0 0102	0.0031	0.1
0.001	0.0081	0.0041	0.1
0.0001	0.0037	0.0022	1.0
0.00001	0.0017	0.0033	—
0.000001	0.0008	0.0007	—
0.0000001	0.0000	0.0019*	0.1
	sorbose		
3.0	0.0020	0.0021	
2.0	0.0075	0.0036	0.1
1.0	0.0074	0.0027	1.0
0.1	0.0072	0.0036	0.1
0.01	0.0076	0.0050	0.1
0.001	0.0045	0.0030	1.0
0.0001	0.0030	0.0029	—
0.00001	0.0031	0.0012	—
0.000001	0.0058	0.0043	—
0.0000001	0.0059	0.0074*	5
	mannose		
6.0	0.0097	0.0024	5
4.0	0.0106	0.0016	5
2.0	0.0124	0.0011	1.0
1.0	0.0132	0.0023	0.1
0.1	0.0111	0.0022	0.1
0.01	0.0074	0.0032	0.1
0.001	0.0036	0.0046*	5
0.0001	0.0043	0.0054*	5
	rhamnose		
1.0	0.0068	0.0004	1.0
0.1	0.0067	0.0005	1.0
	lactose		
1.0	0.0212	0.0159	1.0
0.1	0.0091	0.0012	1.0
	D-arabinose		
0.1	0.0122	0.0004	0.1
	L-arabinose		
0.1	0.0035	0.0036	—

* These values represent the concentration range where water is taken in significantly greater amounts than sugar.

blowfly *Phormia regina* (Hassett, Dethier and Gans, 1950). Sorbose, a hexose, also stimulates the tarsal chemoreceptors (threshold = 0.14 *M*) although it is not utilized. Mannose, a hexose, is extremely poor in stimulating power (tarsal threshold = 7.59 *M*) but is nutritionally highly effective.

The curves describing the ingestion of the three sugars are substantially similar to those obtained by Dethier and Rhoades (1954) with the nutritionally adequate sugars glucose and sucrose. In each case there is a low concentration at which the sugar is not distinguished from water so that equal amounts of solution are taken from each pipette. Then, as the concentration is increased, a point is reached where more sugar than water is imbibed. This point represents a difference threshold. It occurs at a lower concentration than the tarsal acceptance threshold obtained by standard procedures. As the concentration is further increased there is an increase in the volume of solution imbibed until a maximum intake is reached, after which there is a marked decrease. A cursory examination of the curves reveals no relation between the volume intake and either the nutritional value or the relative stimulating effectiveness. Of the three sugars, the maximum intake is greatest for fucose and least for sorbose. None is consumed in as great quantities as glucose or sucrose.

Another characteristic of these curves is an inversion at very low concentrations where water may be taken in preference to sugar. With fucose, sorbose, and mannose the inversion occurs at 1×10^{-7} *M*, 1×10^{-7} *M*, and $1 \times 10^{-3} - 1 \times 10^{-4}$ *M*, respectively. Bimodal preference-aversion relationships of sugars were first noted by Beck (1956) in studies of the larvae of the European corn borer (*Pyrausta nubilalis* Hbn.). A re-examination of the raw data of Dethier and Rhoades (1954) reveals similar relationships. The meaning of rejection at low concentrations is not at all clear.

Individual Intake

When measurements were made of the volume of different concentrations of sugars imbibed by a single fly at one feeding (Table II) and the values plotted as a function of the concentration, the resulting curves differed in several important respects from the customary preference-aversion curves (Fig. 3). With the exception of fucose and sucrose there was no evident tendency for intake to decrease at high concentrations. There was, however, a marked tendency for intake to reach a plateau. On the other hand, regardless of the procedure employed for measuring intake, the weight of sugar consumed increased throughout the entire concentration range. There is no indication that the flies regulate the quantitative intake of sugar.

In comparing individual feeding curves with preference-aversion curves based upon four days of feeding the further difference is noted that the volume intake, while approximately the same in both experiments at high concentrations, at low concentrations is much smaller when measured individually than when measured in a two-choice situation. The fact that one experiment involves a two-choice situation while the other involves no choice has no bearing on the results because Dethier and Rhoades (1954) have shown that intake is the same in one-choice and two-choice situations. It seems possible to explain the difference on the basis of gustatory thresholds and behavior as affected by feeding. Earlier work (*cf.* Dethier and Chadwick, 1948) indicated that feeding elevates taste thresholds, and it seems

reasonable to assume that the greater the ingestion of sugar the longer the taste threshold remains elevated (this assumption is borne out by experiments, soon to be published, on the determinants of taste threshold in *Phormia*). Furthermore, present data show that in general the volume ingested at a single feeding is a direct function of the stimulating effectiveness of the test solution. Hence, it might be expected that in preference-aversion experiments, after once feeding on 1.0 or 2.0 *M*

TABLE II

Amounts of various sugars ingested at a single feeding

Sugar	Molar concentration	Number of animals	Mg./fly	Ml./fly $\times 10^3$	Duration* (sec.)	Rate ml./sec. $\times 10^3$	Approximate viscosity (centipoises)
Sucrose	2.0	20	8.96	13.0	90	14	—
	1.0	30	4.78	13.9	47	30	—
	0.5	10	1.80	10.5	43	24	—
	0.25	10	0.440	7.05	36	20	—
Glucose	2.0	15	4.92	13.7	61	26	—
	1.0	35	2.27	12.6	44	30	—
	0.5	15	0.820	9.11	38	25	—
Mannose	4.0	15	6.49	9.02	51	18	—
	2.0	15	2.97	8.25	40	21	—
	1.0	10	1.12	6.20	38	16	—
	0.5	10	0.268	2.98	25	12	—
Fucose	1.0	50	0.843	5.14	30	20	—
	0.5	15	0.580	7.08	32	35	—
Lactose	1.0	15	0.903	2.82	18	18	—
Sorbose	3.0	10	1.93	3.58	—	—	—
	2.0	20	1.09	3.04	—	—	—
	1.0	10	0.168	0.934	—	—	—
	0.5	6	0.0481	0.534	—	—	—
Sucrose	1.0	10	4.62	13.5	54	25	2.75
Sucrose 1.0*M* in:							
Glycerol	2.2	10	5.20	15.2	60	25	4.29
	5.4	10	2.86	8.34	50	17	7.79
	8.7	4	2.20	6.45	55	12	48.5

* Duration times were recorded for fewer flies than were employed in ingestion determinations.

sugar, the fly would not respond to the solution again for some time when it is encountered; and, furthermore, that when again ingested the solution will be taken in far lesser quantities as a result of the partially elevated threshold. Moreover, the number of encounters with the sugar solution is markedly reduced with flies feeding on 1.0 or 2.0 *M* sugar, since they are almost completely inactive for some time after ingestion of a large sugar meal. When 0.1 or 0.01 *M* sugar solutions are employed for preference-aversion tests, the post-ingestion duration of threshold elevation, the

period of quiescence, and the interval during which response fails upon contact with the solution are all shortened relative to the higher concentrations. The frequency of feeding is thereby increased. Thus may be explained the discrepancy of a higher daily intake of 0.1 *M* than 1.0 *M* sucrose, although at a single feeding much more is taken of the higher concentration.

The action of the above factors is again seen when the raw data of the preference-aversion curves of Dethier and Rhoades (1954) are analyzed on a day-to-day basis. It was found that curves based solely on the first 24-hour intake were displaced to the right, that is, the maximum intake occurred at very high concentrations. For subsequent 24-hour periods the intake of high concentrations drops while that of low concentrations gradually increases (see Dethier and Rhoades, Fig. 2).

The expectation of more frequent feeding on 0.1 *M* than 1.0 *M* sucrose was confirmed by automatic recordings of preference-aversion behavior. During the first eighteen hours of recording, 791 drinks were taken from 0.1 *M* sucrose and only 236 from 1.0 *M*. During the same period there were in addition 1,336 tentative drinks or taste samples of 0.1 *M* as compared with 898 of 1.0 *M*. The duration of drinking was approximately the same with each concentration; however, the volume imbibed per drink of 1.0 *M* was slightly more than twice that of 0.1 *M*. The rate of intake was, therefore, greater in the case of 1.0 *M*. It was also noteworthy that over the entire 18-hour period there was no marked decrease in the number of drinks of 0.1 *M* per hour, but the number of drinks of 1.0 *M* per hour had decreased by 80% at the end of 12 hours. The number had reached 0 at the end of 17 hours.

Sugars Paired with Each Other

In all of the foregoing choice experiments the test sugar was paired with water. In the following experiments sugars were paired with other sugars at many different concentrations. The results are summarized in Table III. From a perusal of these data it may be seen that the results are in general agreement with what might have been expected from an examination of Figure 2. For example, it might have been predicted from Figure 2 that more of 1.0 *M* mannose than of 1.0 *M* fucose would be ingested because the curve for fucose is displaced to the left relative to the mannose curve. The prediction was verified when the two solutions were actually paired (Table III). Similarly, the relative volumes imbibed in other two-choice tests are in general agreement with the basic preference-aversion curves. On the other hand, the *absolute* volumes are not the same in the two types of experiments. Such a discrepancy is to be expected, because volume intake is dependent not only on the concentration of the test solution but on the concentration and identity of all other compounds to which the insect is simultaneously exposed. The total situation is the determinant. For example, it had previously been found by Dethier and Rhoades that the less preferred of two sugars in a paired test was treated as though it were water regardless of how much of it might have been ingested when it was presented alone. In every case here, with the exceptions of 1 *M* mannose paired with 1 *M* sorbose and 0.5 *M* mannose paired with 0.5 *M* sorbose, the same is true. The less preferred member of the pair is ingested at approximately the same level as water (*cf.* Tables I and III). Consequently, the sum of the two volumes ingested in a paired test is generally less than the sum of volumes of each sugar which would have been ingested when paired with water, unless, of course, the less preferred is

TABLE III

Volumes (ml./fly/24 hrs.) ingested when different sugars are paired (preferred sugar underlined)

No.	Solutions paired			Significance at 1% level
1	1.0*M* mannose 0.0077	vs.	1.0*M* fucose 0.0007	+
2	1.0*M* mannose 0.0112	vs.	1.0*M* sorbose 0.0054	+
3	1.0*M* mannose 0.0121	vs.	0.1*M* fucose 0.0045	+
4	1.0*M* mannose 0.0154	vs.	0.1*M* sorbose 0.0014	+
5	0.5*M* mannose 0.0113	vs.	0.5*M* sorbose 0.008	+
6	0.1*M* mannose 0.0017	vs.	0.1*M* fucose 0.0138	+
7	0.1*M* mannose 0.0065	vs.	0.1*M* sorbose 0.0084	−
8	0.01*M* mannose 0.0074	vs.	0.0001*M* fucose 0.0046	+
9	0.001*M* mannose 0.0048	vs.	0.0001*M* fucose 0.0059	−
10	0.1*M* fucose 0.0129	vs.	0.1*M* sorbose 0.0026	+
11	1.0*M* fucose 0.0000	vs.	1.0*M* sorbose 0.0034	+
12	0.01*M* fucose 0.00294	vs.	0.01*M* sorbose 0.00140	+
13	0.1*M* D-arabinose 0.0143	vs.	0.1*M* L-arabinose 0.0043	+

being tested at a concentration at which it is not normally consumed more readily than water. In this last case the total consumption in the paired test would equal the sum of the two sugars tested individually.

In previous pairing of sucrose with glucose and sucrose with sucrose the volume intake of the preferred member was greater than in sugar-water pairs when the concentration in question fell at the peak of the preference-aversion curve, less if it fell on the ascending limb (*i.e.*, low concentrations) of the curve, and equal if on the

descending limb. In the tests reported here the volume intake of the preferred sugar in a pair generally equalled its intake when paired with water when the concentration in question fell at the peak of the preference-aversion curve.

Both sets of data (Tables I and III) suggest very strongly that volume intake is under sensory control, that is, that the stimulating effectiveness of a solution determines how much of it will be imbibed. Several aspects of the two-choice data underline the importance of the sensory rather than the nutritional characteristic of the sugar in regulating volume intake. Line 6 of Table III indicates a preference for 0.1 *M* fucose (non-nutritional) over 0.1 *M* mannose (nutritional). This result clearly indicates the choice of a stimulating sugar over a poorly stimulating one. The choice of 0.1 *M* fucose over 0.1 *M* sorbose (line 10), both sugars being non-nutritional, reflects the superior stimulating effectiveness of fucose at this level of concentration. The relative intake of two sugars at concentrations represented on the ascending limbs of the preference-aversion curves appears to be sense-controlled, the more stimulating sugar always being preferred (lines 4, 5, 6, 8, 10, 12). This conclusion is in agreement with the findings of Dethier and Rhoades (1954) relative to the intake of glucose and sucrose.

When comparisons are made which involve concentrations on the descending limbs of the preference-aversion curves, stimulating effectiveness alone is apparently no longer the sole controlling factor; hence, comparisons at these levels are more complex (lines 1, 11). For example, the preference for 1.0 *M* mannose over 1.0 *M* fucose (line 1) does not result simply from the superior stimulating effectiveness of mannose, for indeed fucose is the more stimulating; instead, the preference undoubtedly reflects a negative factor causing the decline in fucose intake (*cf.* Fig. 3) as being responsible for the preference of mannose in the two-choice situation.

Role of Sensory Systems

The foregoing results clearly implicate the sensory systems. There are three chemosensory systems (exclusive of olfaction) definitely known to be involved in the feeding behavior of *Phormia;* namely, the tarsal chemoreceptors, the labellar hairs, and the interpseudotracheal papillae (Dethier, 1955). The first two mentioned have been studied to a greater extent than the papillae, and most of the remarks regarding stimulating effectiveness in the foregoing section have been based on information so derived. However, on the basis of these studies alone mannose should not be imbibed at all, and certainly its preference-aversion curve should not fall between that of fucose and sorbose.

The difficulty was resolved by the discovery that mannose, while poorly stimulating to tarsi and labellum, was an effective stimulus for the papillae. Its effectiveness at this site explains quite satisfactorily other difficulties encountered in the foregoing section. Mannose is obviously accepted at high concentrations in preference to sorbose, and in preference to water because of its stimulating effect on the papillae. Even though it does not stimulate the tarsal and labellar hairs, except at very high concentrations, it gains access to the papillae as a result of the fly's extending and probing with its proboscis in its normal exploratory behavior and in the course of ingesting to satisfy its need for water.

The discovery of the stimulating effectiveness of mannose on the papillae led to a series of tests in which other selected sugars were applied to the three chemosen-

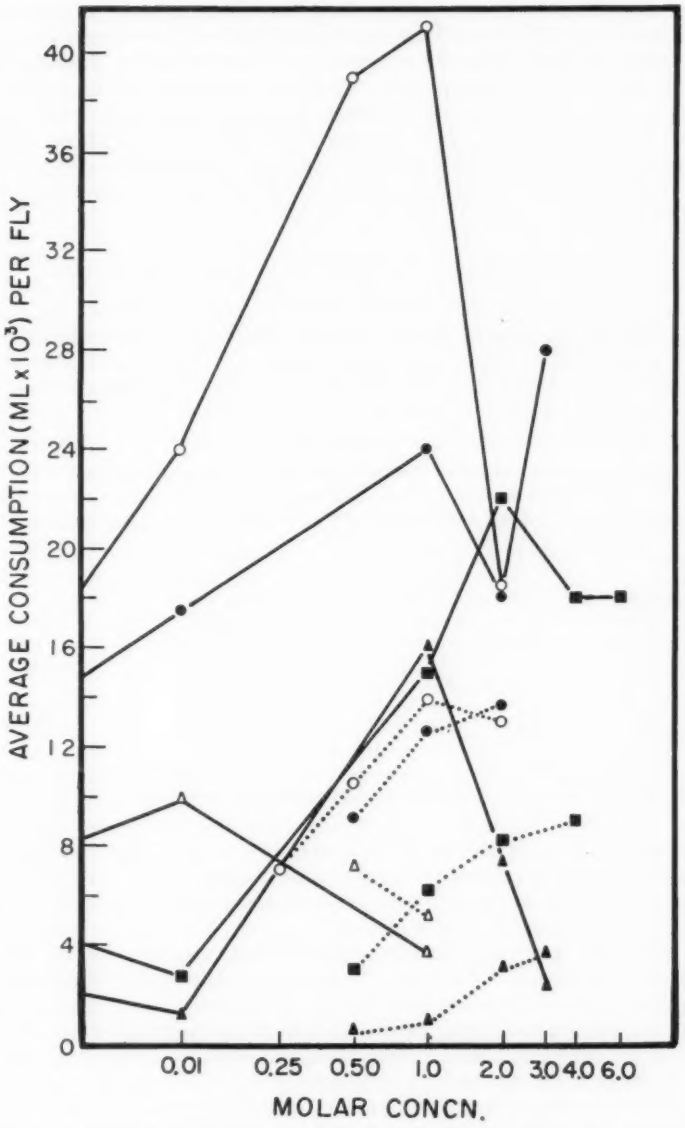

FIGURE 3. Comparison of ingestion measured by single feeding and by preference-aversion intake during the first twenty-four hours. Solid line, preference-aversion; dotted line, single feeding. ○ = sucrose, ● = glucose, ■ = mannose, △ = fucose, ▲ = sorbose.

sory systems. The results are given in Table IV. The most surprising result concerned L-arabinose, which was found to act as a repellent to the papillae even though it is acceptable in terms of its effect on tarsal and labellar hairs. This characteristic of L-arabinose was most unexpected. Clearly it stimulates the tarsal and labellar hairs, as a result of which the fly is moved to extend its proboscis and commence feeding. However, as soon as the solution comes into contact with the papillae, ingestion ceases abruptly. D-arabinose, by contrast, is acceptable to all three chemosensory systems and is consumed in appreciable quantities even though it is not utilized (Table I).

Relation Between Intake and Nutritional Value

From experiments in which different sugars were paired there were already indications that the stimulating rather than the nutritional characteristic of a sugar played a major role in regulating volume intake (*cf.* line 6 of Table III). The minor importance of nutritional factors, at least under experimental conditions, is

Table IV

Effectiveness of selected sugars in stimulating the three chemoreceptive systems of Phormia

Sugar	Tarsal threshold (molar)	Labellar hairs	Interpseudotracheal papillae
fucose	0.087	+	+
sorbose	0.140	+	+
mannose	7.59	−	+
D-arabinose	0.144	+	+
L-arabinose	0.536	+	R
D-xylose	0.440	+	
L-xylose	0.337	+	−
rhamnose	−	−	−
ribose	−	−	
lactose	−	−	−

(+ stimulating, − non-stimulating in all concentrations, R rejected)

revealed further by comparisons of the results of preference tests with sugar mixtures and the capacity of these mixtures for sustaining life. Two examples serve to illustrate the point, the behavior of flies with respect to glucose and D-arabinose and with respect to glucose and rhamnose.

Glucose alone at a concentration of 0.1 *M* supported life for 14 days (50% mortality); D-arabinose, for 3.5 days; a mixture containing 0.1 *M* glucose and 0.1 *M* D-arabinose, for 5.5 days. Survival on water alone averages three days (*cf.* also Hassett, Dethier and Gans, 1950). Yet in preference tests where glucose was paired with the non-nutritional mixture, flies consumed greater quantities of the mixture. Similarly, a mixture of 0.1 *M* glucose and 0.1 *M* rhamnose, which supported life for 8 days as compared to 3.5 days for rhamnose alone and 14 days for glucose alone, was consumed in greater quantity than glucose alone in a paired test (Table VII). Rhamnose paired with water was preferred slightly (Table I).

From these results it would appear that choices were made solely on the basis of the stimulating effect. There is no indication that either D-arabinose or rham-

nose is repellent, since each is in fact preferred to water. While neither interferes with the stimulating effect of glucose on sense organs (Table V), both either are toxic or block glucose utilization.

Intake of Mixtures of Sugars

Sometimes the acceptability of compounds of very low stimulating power cannot be demonstrated in a two-choice test with water or by simple acceptance threshold determinations. Accordingly, the ruse has frequently been employed of mixing two sugars in order to detect suspected additive or repellent properties. Kunze (1927) and von Frisch (1935), for example, found that sugars which were acceptable to the honeybee were strictly additive. Unfortunately the technique is deceptively simple, and the results cannot always be relied upon to give the desired sensory information because of the occurrence of two phenomena which have not been given due consideration. These two are synergism and inhibition. They can be demonstrated most easily and convincingly by measuring tarsal acceptance thresholds to sugars and sugar mixtures. They also occur at labellar hairs. Tests for inhibition and synergism have not been made with interpseudotracheal papillae,

Table V

Examples of inhibition revealed by ascertaining the effects of sugar mixtures on tarsal thresholds

Sugar	Effect	Sugar affected
mannose	inhibits	fructose
	does not affect	glucose, sucrose, fucose, maltose
sorbose	inhibits	glucose, fructose
fucose	does not affect	glucose, fructose
rhamnose	does not affect	fucose, glucose
	inhibits	fructose
D-arabinose	does not affect	glucose
mannitol	does not affect	fructose

but the occurrence of the phenomena at other sites indicates that an additive effect of sugars cannot be assumed as a matter of course.

For example, the median acceptance threshold for fructose is 0.0058; for glucose, 0.132; for an equimolar mixture of the two, 0.0078. In other words, the concentration at which the mixture is stimulating represents 0.0039 *M* glucose and 0.0039 *M* fructose. Even were the two sugars simply additive, they would not be expected to stimulate at this level. The fact that they do stimulate implies synergism. Mannose, on the contrary, when added to fructose inhibits it, that is, causes a ten-fold rise in the fructose threshold. This effect is not due to repellence because, for *Phormia*, mannose is preferred to water in all concentrations above threshold. Furthermore, mannose has no effect on such sugars as glucose, sucrose, or maltose.

The results of threshold tests with other mixtures are summarized in Table V. That the effects observed represent inhibition rather than repellence is further confirmed by the action of sorbose. Sorbose is stimulating in its own right, yet it causes an increase in the thresholds of glucose and fructose when mixed with them. Its action is revealed clearly in the following representative results (Table VI) where the per cent response of a sample of flies to various concentrations of glucose and of sorbose is compared to their response to a series of solutions which contain

0.5 moles of sorbose. In this case the stimulating effect of the mixture at low glucose concentrations stems entirely from the sorbose which is present. At higher concentrations of glucose, when the same amount of sorbose is present, there is little change in the stimulating effectiveness. Not only do the two sugars fail to add, but the stimulating effect to be expected of the high concentrations of glucose is absent. When, therefore, a smaller volume of a mixture of sugars is ingested than of either of the constituents alone, the result cannot always be ascribed to repellence, especially when both constituents can be shown in other tests to be preferred to water.

Galun (1955) has reported that all of the following sugars are repellent to *Musca domestica:* D-xylose, L-arabinose, ribose, rhamnose, and sorbose. This conclusion is based, however, on the fact that the addition of any of these to an acceptable sugar causes a lowering of intake. Unless the sugars can be shown to have a repellent effect when compared with water, the possibility of inhibition cannot be overlooked.

In the present studies some of the results of preference tests with sugar mixtures can be understood in terms of inhibition. For example, the volume intake of a mix-

TABLE VI

Effect of sorbose on glucose threshold

Molar concen. of glucose solutions	0.0625	0.125	0.25	0.50	1.0
Per cent response	0	5	15	50	80
Molar concen. of glucose solutions containing 0.5 *M* sorbose	0.0625	0.125	0.25	0.5	1.0
Per cent response	45	50	60	50	65
Molar concen. of sorbose solutions	0.0625	0.125	0.25	0.50	1.0
Per cent response	5	20	45	60	80

ture of mannose and glucose is greater than that of glucose alone, as would be expected (Table VII). In contrast, the intake of a mannose-fructose mixture, as compared with fructose alone, is not so great as would naturally be expected. Similarly with mixtures containing rhamnose there is a large increase in volume intake where the other sugar is glucose but no appreciable increase where the other sugar is fructose or sucrose (Table VII). This finding is in agreement with the threshold data (Table V) which indicate that rhamnose inhibits fructose but not glucose.

The situation with regard to sorbose is not so clear although there is a tendency for the intake of sorbose mixtures to be less than expected on a purely additive basis. Such a result would agree with the postulated inhibitory effect of sorbose on glucose and fructose. It must nevertheless be emphasized that the effect of sugar mixtures on the papillae is not known, so that the results obtained in preference tests of mixtures cannot be fully interpreted in terms of demonstrated inhibition at tarsal and labellar sites alone.

The difference between repellence and inhibition, at least with *Phormia,* is a real one. Since the tarsal and labellar hairs of *Phormia* have been shown to con-

sist of two receptors, one of which mediates rejection and one of which mediates acceptance (Dethier, 1955), a compound which is repellent might be expected to stimulate the rejection receptor while a compound which is an inhibitor might be expected to prevent stimulation of the acceptance receptor by interfering with the action of a stimulating compound on that receptor.

The only comparable study of mixtures on another insect is that of Wykes (1952), who measured ingestion of single sugars and mixtures of sugars by the honeybee. Although not explicitly stated, the experiment tested the hypothesis that the volume ingested of the four sugars examined, singly and in mixtures, was related to concentration by the formula $V = a + C$ where V is volume ingested at concentration C. For all four sugars, then, there was assumed to be a linear relationship between volume ingested and concentration, with a slope of unity and an intercept depending upon the sugar involved. Since, however, the units of volume

TABLE VII

Comparison of intake of mixed solutions with that of water or single sugars in a two-choice test

Concentration of each sugar in mixture	Vol. consumed ml./fly/24 hrs.	Water or sugar	Vol. consumed ml./fly/24 hrs.
0.05 *M* fucose and 0.05 *M* sorbose	0.0125	water	0.0019
0.5 *M* fucose and 0.5 *M* sorbose	0.0116	water	0.0030
0.05 *M* fucose and 0.05 *M* mannose	0.0213	water	0.0025
0.5 *M* mannose and 0.5 *M* sorbose	0.0184	water	0.0018
0.1 *M* fructose and 0.1 *M* mannose	0.0234	0.1 *M* fructose	0.0130
0.05 *M* glucose and 0.05 *M* mannose	0.0090	0.1 *M* glucose	0.0030
0.1 *M* glucose and 0.1 *M* mannose	0.0228	0.1 *M* glucose	0.0090
0.05 *M* glucose and 0.05 *M* sorbose	0.0099	0.1 *M* glucose	0.0162
0.05 *M* fructose and 0.05 *M* sorbose	0.0220	0.05 *M* fructose	0.0160
0.05 *M* glucose and 0.05 *M* rhamnose	0.0260	0.05 *M* glucose	0.0130
0.1 *M* glucose and 0.1 *M* rhamnose	0.0160	0.1 *M* glucose	0.0070
0.05 *M* fructose and 0.05 *M* rhamnose	0.0120	0.05 *M* fructose	0.0130
0.1 *M* fructose and 0.1 *M* rhamnose	0.0170	0.1 *M* fructose	0.0140
0.05 *M* sucrose and 0.05 *M* rhamnose	0.0180	0.05 *M* sucrose	0.0230
0.1 *M* glucose and 0.1 *M* D-arabinose	0.0210	0.1 *M* glucose	0.0080
0.05 *M* glucose and 0.05 *M* D-arabinose	0.0130	0.1 *M* glucose	0.0160

employed were arbitrary and apparently were changed from one concentration to the next, this hypothesis was not tested directly; it was implicitly assumed in the analysis of ingestion of mixtures. The experiments on mixtures consisted of measuring the volume ingested of a solution containing equal proportions by weight of two to four sugars with a total sugar concentration of $x\%$ and testing the significance of the difference between this value and the average of the volumes ingested of each of the component sugars at $x\%$. For example, the volume ingested of a solution containing 8.5% sucrose and 8.5% glucose was compared with half the sum of the volumes ingested of 17% glucose and 17% sucrose. It was found, rather surprisingly, that the calculated and measured figures were not significantly different; hence, volume and concentration are linearly related, with a slope of unity for sucrose and glucose within the concentration range 17.1 to 51.3%. Similar experiments indicate that the same relationship is true for maltose. Furthermore, with one ex-

ception, these sugars in the mixtures tested are neatly additive in their effect on ingestion. The one exception was the glucose-sucrose-fructose mixture, of which more was ingested than was predicted (*i.e.*, there was synergism). This may reflect the synergism noted above on the tarsal threshold of *Phormia* for a mixture of glucose and fructose.

The data for *Phormia* relating volume and concentration, whether for intake at a single feeding or preference-aversion experiments, never present so simple a picture as Wykes's results. The only similarity may be the striking parallelism (with the exception of fucose) of volume increase from low to the optimum concentrations on a semi-log plot of ingestion at a single feeding (Fig. 3). Preference-aversion experiments on ingestion of mixtures probably are not comparable to ingestion as measured by Wykes; clearly, in the former case simple additivity of sugars in a mixture is not the rule.

The Feeding Reaction

Initiation of feeding. From the foregoing experimental facts and all other available information one can reconstruct, at least in part, the behavior pattern of the normal feeding reaction insofar as it is now known.

The normal pattern consists essentially of extension of the proboscis, spreading of the labellar lobes, sucking, and regurgitation. Apparently any one of three factors may initiate proboscis extension: (1) olfactory stimuli operating primarily through the antennae; (2) taste and possibly tactile stimuli operating through the tarsal receptors; (3) internal factors causing extension spontaneously. In the presence of vapors of an attractive nature a fly will extend its proboscis (*cf.* also Minnich, 1921). If the antennae are amputated, this faculty is impaired. Water (if a fly is thirsty) or specific carbohydrates can stimulate the tarsi with a resultant proboscis extension. In the absence of any specific external stimuli the fly will frequently repeatedly extend its proboscis in an exploratory manner.

The proboscis having been extended in response to any one or combination of these clues, the first parts which come into contact with the substrate are the long hairs of the aboral labellar surface. If the stimulus now received is favorable, the labellar lobes are opened, thus presenting the oral surface to the food. Sucking then commences. The labellar hairs, therefore, can regulate spreading of the lobes and sucking. They can also regulate extension, although under natural conditions it must be quite unusual for the hairs of the retracted proboscis to be stimulated. It could well be that in the event of the omission of an initial step in the normal sequence of stimulation, *e.g.*, stimulation of the labellar hairs before the proboscis is extended, the hairs trigger the missing step, in this case extension, before initiating the remaining steps. Control of the hairs over sucking is easily demonstrated. If, in a fastened fly, a drop of liquid just at the threshold of rejection is placed on the open labellum, it remains undisturbed, and the fly regurgitates into it. Surface tension prevents the fly from closing the labellum, and the feet cannot be employed to remove the drop because they are fastened. If now a single labellar hair is stimulated with a concentrated sugar solution (*e.g.*, 1 *M* sucrose), the drop, diluted with regurgitated fluids, is immediately swallowed.

Having opened the labellar lobes and commenced swallowing, the fly would no longer be in complete sensory control of the situation were it not for the interpseudotracheal papillae. Once the labellar lobes are opened the majority of the aboral

hairs are no longer in contact with the solution. Even if they had been, the speed with which they adapt would certainly prevent a continual input from sugar stimulation from reaching the central nervous system. There is ample evidence that the papillae supply this defect.

Feeding can be monitored at four levels. If an odorous component of food attains a repellent level of concentration, feeding may be inhibited although ordinarily feeding will not have commenced under these conditions. Secondly, if the tarsal receptors are stimulated by unacceptable compounds, feeding is ordinarily stopped and the proboscis withdrawn. This reaction is, of course, the basis of all measurements of tarsal rejection thresholds. Thirdly, if the labellar hairs are affected by adverse stimuli, feeding stops. Fourthly, if the papillae are stimulated by unacceptable compounds, feeding is terminated.

As might be expected, these various levels of control are finely balanced. The coördination of sensory input from all of the receptor systems involved is extremely important for the proper accomplishment of feeding. Consider, for example, the relation between tarsal receptors and those on the mouthparts. Normally a fly will not commence feeding on a solution which has first been rejected by the tarsi. However, if arrangements are made to stimulate tarsi and mouthparts simultaneously with different solutions, the tightness of control of each system over feeding can be assessed. Application of sugar, however concentrated, on the tarsi will not cause feeding if a critical concentration of NaCl is placed on the labellum; but a low concentration of NaCl can be found which will be imbibed when the tarsi are stimulated with sugar, even though this salt is refused in the absence of tarsal stimulation. Conversely, concentrated NaCl on the tarsi will not prevent imbibition of sucrose applied to the labellum. The mouthparts, as might be expected, exert a tighter control.

On the mouthparts themselves the actions of the labellar hairs and interpseudotracheal papillae are usually coördinated. Experimentally either can be stimulated alone. The papillae alone are stimulated by inserting a micropipette between the closed labellar lobes or by rendering the hairs inoperative through waxing. The papillae are extremely sensitive to NaCl, and the application of salt by pipette causes an immediate cessation of feeding. However, it is sometimes possible to force salt imbibition by simultaneous stimulation of labellar hairs with concentrated sucrose. Swallowing is accomplished with great hesitation on the part of the fly if the salt solution is at all concentrated. Conversely, if the hairs are stimulated with NaCl while the papillae are stimulated with sucrose, feeding can be stopped, albeit somewhat slowly and temporarily. From the results of these two experiments it would appear that the papillae exercise tighter control over actual feeding than do the labellar hairs. The behavior of the fly toward L-arabinose confirms this. The hierarchy of command over sucking in ascending order is tarsi, labellar hairs, interpseudotracheal papillae. For proboscis extension and spreading of the labellar lobes, it is tarsi, labellar hairs. Stimulation of the papillae seldom causes proboscis extension or spreading of the lobes so that by means of a micropipette a fly can be induced to feed without extending its proboscis or expanding the labellum. In every case mentioned above the relative concentrations of the opposing stimuli are extremely critical insofar as the nature of the final response is concerned.

Control of volume intake. Although the various chemoreceptors generally work in harmony to regulate the economy of feeding response, the imbibition of liquids is

only the beginning of a longer and more complex chain of events. Once the insect has begun to feed, it obviously does not continue indefinitely. Assuming that the substance being eaten or drunk is an acceptable one and that its stimulating effect (odor or taste) initiated feeding, what are the factors which ensure continuance of feeding and control of volume intake? It seems unlikely that the initial stimulation is alone sufficient to supply momentum for continued feeding without itself continuing, or, in other words, that feeding once started continues automatically until shut off. It is more probable that there is an additional factor which drives continuous feeding and another which terminates it.

Odorous foods not only can supply the initial stimulus but can also continue to stimulate for the duration of feeding. With odorless foods such as sugars, uninterrupted stimulation is also possible. If the fly is standing in sugar, the tarsal receptors can supply a continuous sensory input to the central nervous system until they become adapted. The principal stimulation from the mouthparts during feeding originates at the interpseudotracheal papillae because most of the labellar hairs are no longer in contact with the solution once the lobes have been spread. Even if the labellar hairs were in contact with the sugar, they adapt very rapidly. An experiment can be designed to show that, in the absence of any stimulation except that from the labellar hairs, complete adaptation of these hairs brings an end to feeding. For example, a fly which is not thirsty can be made to drink water if one or more of the labellar hairs are stimulated with sugar. Adaptation of the hair or hairs being stimulated causes feeding to cease, whereupon stimulation of different hairs which are still sensitive results in resumption of swallowing. From this result it would appear that a continual sensory input is indeed essential to uninterrupted feeding. Even stimulation of the tarsal receptors can drive feeding, and one way to force flies to imbibe non-stimulating fluids (*i.e.*, those which are neither acceptable nor repellent) is to apply sucrose to the legs. For many of the insects in which feeding reactions have been studied the prerequisite of sensory input is the rule (*cf.* Dethier, 1953).

Under natural circumstances a fly does not feed to full capacity upon first contact with an acceptable food but rather takes repeated samples. This behavior is graphically demonstrated by automatic recording (Fig. 1). In this way each new extension of the proboscis places the labellar hairs again in contact with the solution for fresh stimulation which imparts renewed impetus to feeding. At some point in the proceedings, however, feeding finally ceases; a definite quantity has been consumed. This volume is not constant but depends upon the hunger state of the fly, the nature of the food, and its concentration. Clearly neither gut capacity nor carbohydrate requirements immediately controls volume intake (*cf.* also Dethier and Rhoades, 1954). Thus, under normal conditions intake may cease long before the gut is fully extended. Furthermore, an isolated head does not drink equal amounts of all sugars. It takes in, for example, less sorbose than fucose and less fucose than sucrose, indicating control by structures of head alone.

An explanation which conforms most closely to the facts as now known is that intake is shut off by sensory adaptation. As an examination of Table II will reveal, the rate of imbibition and the duration of feeding increase with increasing concentration—up to a point. Since rate does increase with concentration and since maximum rates for different sugars are greatest for the more stimulating ones,

there is reason to conclude that *rate* of intake is related to sensory input. It is highly probable, therefore, that the relationship prevails over the entire concentration range but that at a certain point (where measured rate declines) some negative factor intervenes. Since gram intake never declines nor becomes constant, the negative factor cannot be the amount of sugar or excessive or repellent stimulation. The cause must be sought in some other characteristic of the solutions. Increase in viscosity at high concentrations is one limiting factor. Measurements of rates of intake of a series of glycerol solutions of 1 *M* sucrose showed that rate decreases sharply with relatively small increases in viscosity. This finding is in agreement with the results which Betts (1929) had obtained in experiments with honeybees, where rate of intake declined sharply as concentrations of sugar exceeded 50% by weight. Betts concluded that viscosity was the limiting factor in this concentration range. At lower concentrations, however, she observed little change in rate with change in either viscosity or concentration. For the honeybee, temperature appears to exercise greater control over rate of intake than concentration does.

From the fact that duration of feeding increases with concentration one may infer that adaptation is one factor bringing an end to feeding. This inference is in accord with observed increases in adaptation time with increased concentration (Dethier, 1952). Additional evidence in support of this view derives from the observation that a fly which has ceased to feed on a given concentration may be induced to continue on a higher one and that a fly which has been feeding on a high concentration refuses to continue feeding on a lower one. In this respect isolated heads behave similarly. If the inference is correct, it would appear that flies adapt most quickly to fucose and less quickly to mannose, glucose, and sucrose, respectively, because this is the inverse order of duration of feeding.

Although the immediate cessation of imbibition can be explained in terms of adaptation, peripheral and central, and there is no evidence of action by internal factors at this point, it is almost certain that subsequent intake at various times after feeding to repletion is regulated by internal factors. These factors have been investigated and will be discussed in a latter communication.

Summary

1. The ingestion of sucrose, glucose, fucose, sorbose, mannose, and lactose by the blowfly *Phormia regina* was studied by means of preference-aversion tests conducted for four-day periods; individual feeding tests; measurements of the sensitivity of the different chemoreceptor systems; measurements of volume intake of mixed solutions; and longevity tests.

2. The preference-aversion curves for all sugars studied indicated an increase in volume intake with increasing concentration up to an optimum point, after which there was a decrease in intake. At very low concentrations water was preferred to sugar.

3. Volume intake measured by individual feeding tests did not exhibit a pronounced decline at high concentrations. The difference between this finding and the one noted above resulted from the fact that flies ingested a maximum volume of concentrated solutions during the first visits to the pipette and then gradually ceased feeding altogether, while their ingestion of less concentrated solutions con-

tinued repeatedly over the entire test period. In all experiments the weight of sugar taken increased over the entire concentration range.

4. There is no relation between the amount of sugar taken and its nutritive value.

5. Volume intake is under sensory control. The coördinated actions of three principal chemosensory systems regulate the complete feeding reaction. The intake of mixed solutions depends upon the stimulating effectiveness of the mixture and whether or not any of the components exhibit synergism or inhibition. Some sugars show inhibition but no repellence.

6. The initiation of the feeding reaction is under sensory control. Continuance of feeding is dependent upon continuous sensory input. The rate of imbibition increases with concentration until viscosity begins to exert a restraining effect. The termination of feeding may be brought about by adaptation.

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THE CULTURE OF BRINE ALGAE [1]

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The biological productivity of the solar evaporation ponds used in salt manufacture is apparent even to the casual observer: they are a deep green or rich red. The ecological association in these ponds was the subject of several investigations (Peirce, 1914; Baas-Becking, 1928; Carpelan, 1953). Carpelan concluded that the productivity of the ponds, per unit area, was comparable to that of the ocean in its richer spots. But the ponds, being only half a meter deep, have a much more concentrated plankton community than does the productive zone of the ocean, which is 10 to 15 meters deep.

With the increased interest in recent years in mass culturing of algae for food production, the possibility of utilizing these evaporation ponds for such purposes has been considered. With this in mind, a laboratory study was undertaken on the cultivation of several of the unicellular algae which thrive in the ponds.

Pure cultures were isolated by the procedures described by Pringsheim (1946), with the added advantage of utilizing antibiotics, as suggested by Spencer (1952). The following five algae were studied:

Stichococcus sp.
Platymonas sp.
Dunaliella viridis
Dunaliella salina
Stephanoptera gracilis

These were isolated from the ponds of the Leslie Salt Company, on San Francisco Bay. The author is indebted to this company for many privileges during the course of the study.

Salinity Tolerance

The determination of the range of salinity tolerated by the different algae under study is of primary importance. For this purpose several strains of the algae were inoculated into solutions of artificial sea water of varying concentrations.

Stichococcus strains numbers 1, 2 and 3, *Platymonas* strains 5 and 7, *Dunaliella viridis* strains 6, 8 and 9, *Dunaliella salina* strain 10, and *Stephanoptera gracilis* were studied. The strain numbers refer to the evaporation ponds from which they were isolated. Pond number 1 contained San Francisco Bay water (concentration less than sea water). The successive ponds contained increasing concentrations of brine to NaCl saturation in pond number 10.

Artificial sea water was prepared after Pringsheim (1946). Multiples of the sea water formula were used for preparing the desired concentrations. Concentra-

[1] Study partly supported by a grant from the National Science Foundation to Stanford University.

tions up to ten-fold sea water were prepared; the solutions of concentration of X 5 and above were saturated with respect to the $CaSO_4$, excess solid being filtered off. Dilutions of sea water to ½, 1/10, as well as fresh water, were also used. All solutions were equally enriched with 50 mg. KNO_3, 10 mg. KH_2PO_4 and Hutner's trace elements mixture (Hutner *et al.*, 1950). Five-ml. portions of the various solutions were pipetted into test tubes and equally inoculated. The test tubes were kept in an inclined position under continuous illumination. Light was provided by several fluorescent lights suspended above the test tubes; its intensity was about 150 foot candles.

Growth was estimated by cell counts after 16 days of culture. At this time the tubes in which no growth had taken place were re-inoculated with 0.1 ml. of the culture which was nearest to them in concentration. Thus, if a culture in the tubes of X 7 sea water did not grow, it was inoculated with the growing X 6 culture. The

TABLE I

Relative growth of algal strains isolated from different salinities in media of different concentrations

Strain:	Medium Concentrations												
	0	1/10	1/2	1	2	3	4	5	6	7	8	9	10
Stichococcus sp.													
1.	30%	74%	170%	100%	NT								
2.	T	46%	135%	100%	15%	NT							
3.		T	90%	100%	40%	NT							
Platymonas sp.													
5.	77%	77%	90%	100%	90%	47%	NT						
7.		50%	100%	100%	125%	230%	60%	NT					
Dunaliella viridis													
6.	63%	82%	240%	100%	100%	95%	85%	50%	26%	NT			
8.	NT	34%	?	100%	225%	320%	230%	125%	100%	18%	T		
9.		T	60%	100%	210%	400%	320%	310%	120%	40%	T	T	T
Dunaliella salina													
10.		T	100%	100%	160%	220%	215%	185%	145%	80%	60%	55%	35%
Stephanoptera gracilis													
11.			T	12%	100%	120%	135%	67%	30%	T	T	T	

(Concentrations in multiples of sea water; T and NT refer to trainability or non-trainability of the algae for growth in the specific concentrations. Growth in sea water taken as base value for all strains except *Stephanoptera gracilis* in which X 2 sea water was taken as base value.)

results of these experiments are given in Table I. The results are expressed as per cent of the growth in sea water, except for the values for *Stephanoptera gracilis,* which are expressed as per cent age of X 2 sea water medium. The results of the secondary inoculation are expressed as T (for trainable) and NT (for not trainable).

From Table I it is apparent that the *Stichococcus* strains under study are primarily brackish water organisms. The *Platymonas* sp. appear to be more resistant to salt, although they too did not grow in the highly concentrated brines. The last three algae, all of the Polyblepharidaceae family, tolerate a wide range of salinity. Of these, *Stephanoptera gracilis* did not grow well at concentrations below X 2 sea water.

TEMPERATURE

To determine the temperature range for the organisms under study, the following procedure was adopted. Sea water was used for *Dunaliella viridis, Platy-*

monas sp. and *Stichococcus* sp.; *Dunaliella salina* and *Stephanoptera gracilis* were cultured in X 2 sea water. Media were enriched with nitrate, phosphate and Hutner's trace elements. Portions of sterile media were inoculated with the above mentioned cultures, five-ml. portions being pipetted into sterile test tubes.

Cultures were incubated in thermostatically controlled incubators, with light provided from two 15-watt fluorescent lamps. The test tubes were set on an inclined rack at a distance of 20 centimeters from the light source. Light intensity at the culture tube level was above 100 foot candles. After 10 days of continuous illumination, growth was estimated in the cultures by cell count. The results of the counts are given in Table II. The range of temperatures recorded by Carpelan (1953) for the evaporation ponds of the same region was from 8° to 20° in the winter, and 15° to 30° in the summer.

pH Effect

The effect of pH on the growth of the algae was investigated with the aid of media buffered with "Tris" (1, 3-propendiol-2-amino-2-hydroxymethyl). The pH

Table II

Effect of temperature on growth of brine algae
(Cells per cubic millimeter)

Temperature ° C.	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
8–10°	20	90	2,700	120	4,200
14–16°	200	560	36,000	340	24,000
24–26°	160	640		1,100	47,000
30°	450	1,800	35,000	1,000	40,000
35°	25	40	no growth	1,100	16,000

range between 7 and 9 was investigated since this is the most common value for the sea water brines. Culture media were prepared as before except for the addition of 0.4 *M* "Tris" buffer; pH was adjusted to 7.2, 8.0 or 9.0. Five-ml. portions of the media were pipetted into test tubes and equally inoculated. The test tubes were kept inclined under continuous illumination. Cell counts were made after 9 days of growth. The results are recorded in Table III.

As seen from the table only *Stichococcus* sp. and *Platymonas* sp. show a definite preference for higher pH values. The other three algae do not seem to be very sensitive to pH changes in the range studied.

Phosphate Concentration

The optimum range of phosphate concentration for the algae under study was determined in an experiment in which 5 ml. of inoculated media in test tubes were enriched with graded quantities of phosphate. The media were buffered to pH 8 with .04 *M* "Tris" buffer. Nitrate and micronutrients were supplied as in the previous experiments. Cell counts were made after 10 days of cultivation under continuous illumination. The results are tabulated in Table IV. The optimum

TABLE III

Effect of pH on growth of brine algae

	Original pH	Final pH	Cells/mm.³
Dunaliella salina	7.2	7.5	770
	8.0	8.1	1,300
	9.0	9.1	1,380
Stephanoptera gracilis	7.2	7.8	1,100
	8.0	8.1	740
	9.0	9.2	760
Dunaliella viridis	7.2	7.6	24,000
	8.0	8.0	15,000
	9.0	9.2	20,000
Stichococcus sp.	7.2	7.4	16,000
	8.0	7.9	25,000
	9.0	9.2	69,000
Platymonas sp.	7.2	7.5	570
	8.0	8.0	1,000
	9.0	9.2	2,270

range as seen in the table is in the same range of concentration which is recommended for use with several other marine algae (Koch, 1953; Ketchum and Redfield, 1938; Kylin, 1943).

NITROGEN SUPPLY

The growth of the algae on nitrate or ammonium as the nitrogen source was investigated. These are easily available forms of fixed nitrogen for mass cultivation of plants.

Media were prepared as before, buffered with "Tris" to pH 8.1. Phosphate and trace elements were supplied. Potassium nitrate was added in concentrations of 50 mg./100 ml. NH_4Cl was added in concentrations of 26.5 mg./100 ml. Cell counts were done after two weeks of culture under continuous illumination. In Table V the results of this experiment are summarized.

TABLE IV

Effect of phosphate concentration on growth of brine algae

(Cells per cubic millimeter)

KH_2PO_4 (microgram/ml.)	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
0	340	280	1,300	270	8,500
20	450	630	3,000	520	8,800
40	710	600	2,800	830	12,700
100	540	450	2,400	700	13,700
200	420	300	1,400	730	13,500
500	200	190	1,200	560	12,300

In another experiment, summarized in Table VI, using an unbuffered medium and higher concentrations of nitrate and ammonium (½ mg. N/ml.), the superiority of nitrate over ammonium for growth of all the investigated algae was clearly demonstrated. In this experiment urea was also studied; it was found to be a superior nitrogen source only for *Dunaliella salina*.

The ability of the algae to grow when supplied with organic nitrogenous substances as sole nitrogen source was also investigated. Media were prepared enriched with phosphate and micro-nutrients. The nitrogen source was added to a

TABLE V

Nitrate and ammonium as nitrogen sources for brine algae in media buffered to pH 8.1

(Cells per cubic millimeter)

	Dunaliella salina	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
NO_3	1,700	1,500	4,700	6,000	48,000
NH_4	1,900	2,500	13,000	6,300	28,000

final concentration of 50 micrograms N/ml. The media were buffered with "Tris" to either 7.5 or 9.0. Cell counts were made after 12 days of culture, the results being tabulated in Table VII.

Contrary to expectation, uric acid, which is the main nitrogen form in bird excrement, does not serve as a good nitrogen source for *Stephanoptera gracilis* although this alga is usually found to bloom in high tide pools on rocks which are coated with bird excrement. *Platymonas,* which also blooms under similar condi-

TABLE VI

Effect of nitrogen source on growth of brine algae

(Number of cells per cubic millimeter and final pH values indicated)

Nitrogen source	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
	Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
NO_3	1,000	8.2	1,700	8.9	39,000	8.5	2,000	9.5	20,000	9.3
NH_4	600	6.4	700	5.0	6,000	5.5	400	6.5	10,000	6.9
Urea	1,400	7.8	500	7.5	6,000	7.5	300	8.3	8,000	7.8

tions, can utilize uric acid. *Platymonas* alone was found to grow on all the organic nitrogenous substances studied; this might be a clue to an observation that this alga appeared to coat glassware in which animal or plant materials are kept under running sea water in the laboratory. *Stichococcus* sp. prefers nitrate to uric acid and asparagin; this is in contrast to the strains studied by Ryther (1954) which apparently grow better on uric acid and asparagin than on nitrate.

The experiment was performed at two pH values with the hope of gaining information on the relative availability to the cells of the different ionic forms of the ex-

TABLE VII

Effect of various nitrogen sources on growth of brine algae
(Number of cells per cubic millimeter, and final pH values indicated)

Nitrogen source	Original pH	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
		Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
Nitrate	7.5	870	7.8	1,370	7.9	25,000	7.8	530	7.8	28,000	7.7
	9	1,700	9.0	380	8.9	39,000	9.1	1,100	9.1	70,000	9.1
Uric acid	7.5	880	7.5	0		3,000	7.5	530	7.6	11,000	7.6
	9	1,100	8.7	0		4,500	8.7	1,300	8.9	17,000	8.8
dl-Aspartic acid	7.5	0		0		0		130	7.3	0	
	9	0		0		0		200	8.6	0	
Glutamic acid	7.5	970	7.9	530	7.8	0		500	7.7	0	
	9	530	8.9	270	8.8	0		500	8.9	0	
Asparagine	7.5	210	7.6	120	7.6	0		470	7.7	8,000	
	9	210	8.6	110	8.7	0		1,200	8.9	8,000	
Glycine	7.5	0		0		0		1,000	7.7	0	
	9	0		0		0		1,200	8.9	0	

amined substances. The growth on uric acid was found in general to be pH-dependent in much the same way as growth on nitrate. On the other hand growth on glutamic acid seems to show a reverse pH response in the case of *Dunaliella salina*.

ORGANIC NUTRIENTS

The ability of the brine algae to grow in the dark on several organic substances, as well as the effect of these substances on the growth in light, was investigated. Two organic energy sources were investigated, glucose and acetate. The effect of

TABLE VIII

Effect of organic substances on growth of brine algae
(Cells per mm.^{3})

Medium	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
1. Mineral only	700	970	6,250	630	27,000
2. Glucose	670	980	11,300	430	26,000
3. Acetate	580	870	6,200	770	—
4. YE* Glucose	700	750	9,200	420	16,000
5. YE Acetate	780	720	7,200	970	—
6. BH** Glucose	1,300	1,070	11,500	700	20,000
7. BH Acetate	670	900	9,500	920	—

* Yeast extract.
** Brain-heart infusion.

these was studied alone and in conjunction with complex mixtures such as yeast extract (YE) or brain-heart infusion (BH). The media were enriched with nitrate, phosphate and trace elements, and strongly buffered to pH 8.1 with 0.1 *M* "Tris" buffer. Glucose or acetate were used to final concentration of ½%, brain-heart infusion to .02% and yeast extract to .01%. Five-ml. portions of the inoculated media were pipetted into test tubes. Two equal sets of test tubes were maintained, one under continuous illumination and the other in the dark. The illuminated tubes were counted after 7 days of culture. The results are given in Table VIII. No growth was detectable in the tubes which were kept in the dark for three weeks.

The inhibition of growth of *Stichococcus* by the acetate is remarkable. The inhibitory effect of undissociated acetate on *Chlorella* is discussed by Myers (1951). In our case, however, the concentration of undissociated acetate is very low (about .0003 *M*). *Dunaliella viridis* seems to be stimulated by glucose, while *Platymonas* is stimulated by acetate.

Summary

1. The conditions which the brine algae require for growth were found to be relatively simple. The high temperatures in the pond waters during the summer are well within the tolerance range of these organisms.

2. Simple nitrogenous substances and no organic supplements are required. No need for organic growth factors could be demonstrated under the conditions of cultivation used. Sea water, especially as it concentrates by evaporation, probably contains most of the required trace elements in sufficient quantity. It is possible that for mass cultivation of very dense algal suspensions, supplements of the micronutrients will be required.

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SOME ECOLOGICAL RELATIONSHIPS BETWEEN PHYTO- AND ZOOPLANKTON [1]

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The possibility of increasing human food resources by cultivation of unicellular algae is being rather widely investigated now (*cf.* Burlew, 1953). Two main approaches are considered: closed system cultivation of a pure algal culture under optimum growth conditions, and an open system, utilizing ponds. The closed system has the advantage of providing the maximal rate of photosynthesis and crop yield; however, it is expensive to install and maintain. The open system does not require elaborate installations and has the added advantage of utilizing ponds which may be constructed for a different purpose, algae being a by-product. Of such systems suitable for algal cultivation, sewage oxidation ponds are being studied. The growth of algae in these ponds is beneficial to the oxidation process, and the harvested algae save much of the nitrogen wastes and minerals which are otherwise poured into the sea (Gotaas *et al.*, 1954).

The solar evaporation ponds of the salt industry might also be utilized for this purpose. The natural productivity of these ponds per unit area approaches that of the open ocean (Carpelan, 1953), the difference being that the ponds are only one-half meter deep while the productive zone of the ocean is ten or more meters deep. This gives a very dense standing crop.

One of the economically limiting factors in mass cultivation of algae is their harvesting. In dense algal suspensions the volume of cells is still only a fraction of the total volume. To aid in the harvesting, suggested procedures have been sedimentation by slow settling (Smith, 1953) or flocculation of cells by added alum (Gotaas *et al.*, 1954).

Another possibility is utilizing grazing animals for the purpose of harvesting the algal cells. Raising fish in ponds is an old practice, the growth of plankton being accelerated by various fertilizers. However no attempt is usually made to maintain a maximal rate of production of the primary food, the algae.

In a preliminary investigation of the possibilities of the utilization of the sea water evaporation ponds of the salt industry, several unicellular algae which grow in this environment were studied (Gibor, 1956). We considered the possibilities of converting the algal crop to an animal crop by feeding a zooplankton grazer.

One of the important grazers in the evaporation ponds is the brine shrimp *Artemia*. This organism is easily maintained in the laboratory; its "eggs" (cysts) are readily available and can be kept in the laboratory for many years without losing their ability to hatch. We attempted to study the nutritive value to *Artemia* of several of the unicellular green algae which were isolated from the brines of the evaporation ponds. The algae used were:

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Dunaliella viridis
Dunaliella salina
Stephanoptera gracilis
Platymonas sp.
Stichococcus sp.

Dunaliella salina and *Stephanoptera gracilis* were cultured in sea water evaporated to half its original volume; the other three species were cultivated in sea water.

The feeding experiments were carried out as follows. *Artemia* cysts had been collected two years earlier from salt ponds of the Leslie Salt Company, and kept in a closed jar in the laboratory. Such cysts were suspended in water and centrifuged (in a clinical centrifuge) for several minutes. The light cysts were decanted off as suggested by Dempster (1953). The heavy cysts were re-suspended in merthiolate solution (1 : 1000, in water) for 10 minutes, then centrifuged. The merthiolate solution was decanted off and the cysts washed four times with sterile sea water to get rid of the merthiolate. Finally the sterile cysts were transferred into a one-liter Erlenmeyer flask containing sterile sea water and allowed to hatch.

The sterility of the cysts and larvae was determined by suspending a fraction of the sterilized cysts in sea water enriched with 2% yeast extract-glucose solution and

TABLE I

Size of Artemia salina fed on different brine algae for six days (average length exclusive of caudal furca)

Alga	Sterile culture	Non-sterile culture
Stephanoptera gracilis	2.3 mm.	2.8 mm.
Dunaliella viridis	2.1 mm.	2.8 mm.
D. salina	1.5 mm.	2.3 mm.
Platymonas sp.	1.5 mm.	2.0 mm.
Stichococcus sp.	0.7 mm.	0.4 mm.
None (unfed)	0.2 mm.	0.2 mm.

incubating for several weeks. To further verify their sterility a dense cyst suspension (⅓ ml. eggs in one ml. enriched sea water) was also incubated. The autolysing cysts should supply additional growth requirements for contaminating bacteria. Both these tests were negative and indicated the adequacy of the sterilization method. The larvae were transferred when needed with a sterile Pasteur pipette into a test tube for addition to the algal culture. Ten to 20 larvae were introduced into one liter of a dense algal culture in a two-liter Fernbach flask. The culture was kept under continuous illumination, with continuously bubbling air. After 6 days the cultures were still green, indicating that until this time the quantity of food was not limiting the growth of the animals. At this stage the *Artemia* were harvested by passing the whole solution through a fine plankton net. The animals were fixed in 0.5% formalin in sea water. The control larvae in sterile sea water were dead of starvation at this time.

The results of the feeding experiment are shown in Table I.

Dunaliella viridis and *Stephanoptera gracilis* appear to be superior as foods to *Dunaliella salina* and *Platymonas* sp., while *Stichococcus* sp., is evidently a poor nutrient for *Artemia*. Consistent results were obtained in a second experiment ex-

cept that growth on *Dunaliella salina* in this case was as good as on the other two Polyblepharidaceae.

For ecological purposes it seemed advisable to find whether these results also hold under non-sterile conditions. The apparent superiority of the Polyblepharidaceae might, for example, be due to the absence of a rigid cellulose wall; bacteria (in the *Artemia* gut) might aid in the digestion of the cellulose wall of *Platymonas* and *Stichococcus*. An experiment therefore was conducted under conditions identical to the first except for the use of unsterilized *Artemia* larvae. These results (Table I) show an improved growth in all cultures except *Stichococcus* sp. with the Polyblepharidaceae still showing better growth. Bond (1933) found that *Platymonas* sp. was slightly superior to *Dunaliella viridis* as food for *Artemia*. His criterion was the time in which *Artemia* reached the mating stage: on *Platymonas* sp. the required time was 28 to 29 days, on *Dunaliella viridis* 31 days. However in one of our experiments non-sterile *Artemia*, growing on *Dunaliella viridis*, were found copulating after 13 days.

To investigate whether *Stichococcus* is producing an inhibitor to the growth of *Artemia*, a mixed culture of *Stichococcus* sp. and *Dunaliella viridis* was inoculated with *Artemia* larvae. Good development of the larvae showed that no inhibitor was produced.

Microscopic observation established that the *Artemia* larvae do ingest *Stichococcus;* the deficient growth on this alga is thus not due to the inability of the animal to filter and ingest the smaller cells of this genus.

On the basis of the estimation of the *Stichococcus* crop and the population of *Artemia* in the evaporation ponds Carpelan (1953) concluded that *Artemia* utilizes only a small fraction of the crop of *Stichococcus*. Our results based on laboratory tests corroborate this opinion.

The observations made in the experiment on the nutritive value of algae for *Artemia* aid in understanding the ecological relationships in the series of evaporation ponds.

One of the striking facts observed in the salt ponds is the predominance of *Stichococcus* in brines of relatively low salinity (to about three-fold sea water). Both *Platymonas* sp. and *Dunaliella viridis* can be isolated from such low salinity brines, and both algae grow well in these concentrations. However, they are always overgrown by *Stichococcus*.

The finding that *Artemia* does not grow on *Stichococcus* suggested that the animal might act as a differential filter, ingesting the algae on which it grows well and leaving those on which it can not grow. The possibility of a differential mechanical effect was eliminated by observations on starved *Artemia* put into a *Stichococcus* suspension. As mentioned above, the animals fill their gut with this alga in a few minutes.

To determine whether live cells survive in the fecal pellets, sterile *Artemia* were fed on a pure culture of *Stichococcus*. After feeding for several hours the animals were washed by transferring them into a corner of a Petri dish containing sterile sea water. Use was made of the positive phototropic response of the *Artemia*. The fast swimming animals were collected from the opposite, light side of the dish and transferred to a second dish for repeated washing. After 4–5 such washings the animals were transferred into a depression slide containing sterile sea water and left for several hours. Fecal pellets accumulated in the depression slide. Single pel-

lets were picked with a sterile Pasteur pipette and transferred through a series of sterile sea water droplets. The washed pellets were finally inoculated into test tubes containing several milliliters of sea water enriched with minerals, and kept under continuous illumination.

Test tubes in which growth of algae occurred were examined microscopically to determine whether we were dealing with the same algae as fed to the animal. Ten test tubes so treated were found to contain growing *Stichococcus* cultures. Clearly some cells survived ingestion. An ecological advantage for one algal species over another might be established by even a slight difference in such ease of digestibility in the gut of a non-differentiating filter feeder.

In order to investigate this possibility in a mixed algal population the following experiment was performed. Young growing cultures of *Stichococcus, Dunaliella viridis* and a mixture of both algae were divided into two equal portions of 10 ml. each in 50-ml. Erlenmeyer flasks. Into one flask of each pair about 12 *Artemia* larvae were added. The flasks were kept under continuous illumination.

After ten days the following results were recorded: the *Stichococcus* flasks were both equally green, and growing. No appreciable growth of the larvae had occurred although some were still alive. The *Dunaliella viridis* cultures were entirely different. The flask without the animals was bright green, while the flask containing the animals was completely clear, and with the larvae growing well. The results of the mixed cultures were striking. The flask without the animals was deep green and growth of both algae was obvious. (The presence of *Dunaliella viridis* was easily ascertainable without a microscope since motile cells accumulated on the illuminated side of the flask.) The flask with the animals was not as green as the control flask and no obvious population of *Dunaliella viridis* could be seen by superficial observation. The animals in this culture were alive and growing, but they were not as large as the animals grown on the pure *Dunaliella viridis* culture. In the mixed culture without animals microscopic examination revealed the presence of a large number of both *Stichococcus* and *Dunaliella* cells. In the flask containing the animals, very few *Dunaliella* cells could be seen among the many *Stichococcus* cells. Later observations on the flasks, three weeks after the beginning of the experiment, revealed that the well developed *Artemia,* which had eaten and cleared the *Dunaliella viridis,* were dead, apparently of starvation. There was an indication of fresh growth of *Dunaliella viridis.* The animals were also dead in the dense *Stichococcus* sp. culture. On the mixed culture several living individuals were seen. However they were not as well developed as the animals which grow on the pure *Dunaliella viridis* culture.

A similar experiment was performed with a different zooplankton organism, the copepod, *Tigriopus.* The results were similar to those with *Artemia.* These animals could not utilize the *Stichococcus* cells available to them; *Dunaliella viridis* cells, on the other hand, were readily consumed. The ecological implications of these observations are of considerable importance. In standard oceanographic observations all the phytoplankton is considered as available food to the zooplankton. The curious phenomenon, often observed, of the scarcity of zooplankton in waters rich in phytoplankton, and vice-versa, was explained as due either to overgrazing (Harvey *et al.,* 1935) or to animal exclusion by production of inhibitors (Ryther, 1954). The present study suggests the possibility that certain phytoplankton organisms are not suitable food for some planktonic grazers.

SUMMARY

1. Several planktonic algae from the brines of the sea water evaporation ponds were fed to the brine shrimp *Artemia*. They were found to differ in their nutritive value to this filter-feeding animal. One of these algae, *Stichococcus* sp., could not be utilized by the animal as a food source.

2. Controlled experiments of the effect of filter-feeding *Artemia* and *Tigriopus* on a mixed population of two unicellular algae indicate that the animals are capable of acting as differential grazers. The heavy bloom of *Stichococcus* in the evaporation ponds could be due to the effect of preferential digestion of competing algae by grazing animals.

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FURTHER OBSERVATIONS OF HOMING TERNS

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Among the most promising recent experiments in the field of bird orientation have been those of Kramer (1952, 1953) and Matthews (1953a, 1953b) in which pigeons and Manx shearwaters released in unfamiliar territory oriented approximately towards home while still within view of the release point. This ability to choose the homeward direction within a few minutes after release seems to be lost when the sun is obscured by clouds. We have recently reported homing experiments in which common terns (*Sterna hirundo*) showed a directional orientation by tending to fly southeast whether the home direction was northeast, south, or southwest (Griffin and Goldsmith, 1955). In these experiments terns from breeding colonies in Massachusetts and Maine were removed to inland areas, released singly, and observed with binoculars for as long as possible from two points about half a mile apart. The simultaneous cross bearings thus obtained allowed us to reconstruct the first few minutes, and the first one to two miles, of the birds' flight. The terns showed a consistent tendency to head approximately southeast when the sun was visible; but with heavy cloud cover they seemed to scatter at random. The southeasterly headings were independent of the home direction, of the wind direction, of the time of day, and (except for bodies of water) of local topography. Even though common terns do not seem to be as skillful navigators as the pigeons studied by Kramer and Matthews, the results are of interest because they add to the growing body of evidence pointing to the sun as an important factor in the orientation of birds.

Matthews (1955) has questioned whether these results do in fact represent a directional tendency on the part of the terns rather than "a crude form of homeward orientation." We therefore wished to extend our previous observations by adding a release point at which home would lie in the opposite direction from southeast. A further reason for this additional experiment was our suggestion that the habit of flying southeast when suddenly released in unfamiliar inland territory might be an advantageous one for terns nesting along the eastern coast of the United States since it would bring them quickly back to the coast. Both considerations indicated an experiment to compare the initial headings of terns nesting in the Great Lakes region with those from the Atlantic coast.

Methods

The release point selected for this experiment was the airport at Cortland, New York, about midway between Cape Cod and Detroit. This airport is about 12 miles from the nearest lake of any size (Skaneateles), it has little traffic, and the terrain affords a relatively clear view in all directions. Thirty-two terns were captured late on the afternoon of June 8, 1955 at a nesting colony at Metropolitan

Beach, Michigan (15 miles north of Detroit on the shore of Lake St. Clair); on the morning of June 9, nine others were caught on Penikese Island, Massachusetts. Both groups were transported by automobile in boxes covered so as to prevent the birds from observing their surroundings, and all were released on June 10 between 9:30 A.M. and 6:30 P.M. E.S.T., after 24–48 hours in captivity. The methods of capture and handling were the same as in our previous experiments. The terns were again observed through binoculars mounted on tripods equipped with alidades so that bearings could be noted by an assistant. Three observers participated in this experiment, so that the bearings were taken from the three corners of a triangle with legs approximately 0.4, 0.6, and 0.8 mile in length—the eastern corner being the release point. The terns from the two colonies were released in an ir-

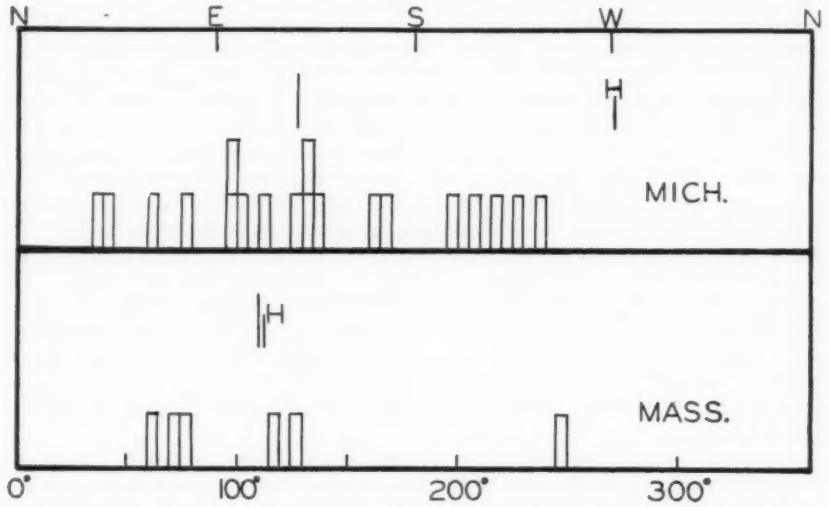

FIGURE 1. Graphic comparison of the initial headings of 25 common terns from colonies in Massachusetts and Michigan released the same day at Cortland, New York. Homeward direction is indicated by an "H", mean heading of each group by an unmarked vertical line.

regular sequence, and each bird was set free only after the previous one had been lost from view by all three observers. No attempt was made to check the homing performance by subsequent observations of the nests, since earlier studies had shown that the homing times of terns are too long to contribute any useful information about the route flown (Griffin, 1943).

We should like to express our gratitude to R. Gibbs, W. Jablonski, Mrs. B. Johnston, W. Nickell, A. Novick, R. Payne, and R. Risebrough whose help in capturing and observing the birds made this experiment possible, as well as to the Office of Naval Research which provided financial support through a research contract with Harvard University.

Observations of Initial Headings

All of the terns were released under clear or partly cloudy skies when the position of the sun was evident, though the sun itself was sometimes temporarily hidden behind a cumulus cloud. The day was warm, and there were numerous updrafts on which the terns tended to climb and soar. We thus lost sight of some birds at heights of several hundred feet above the ground before they had indicated a definite heading away from the release point. Unknown to us at the time the releases were begun, there was also a small pond 1.2 miles to the southwest of the release point which attracted some of the terns.

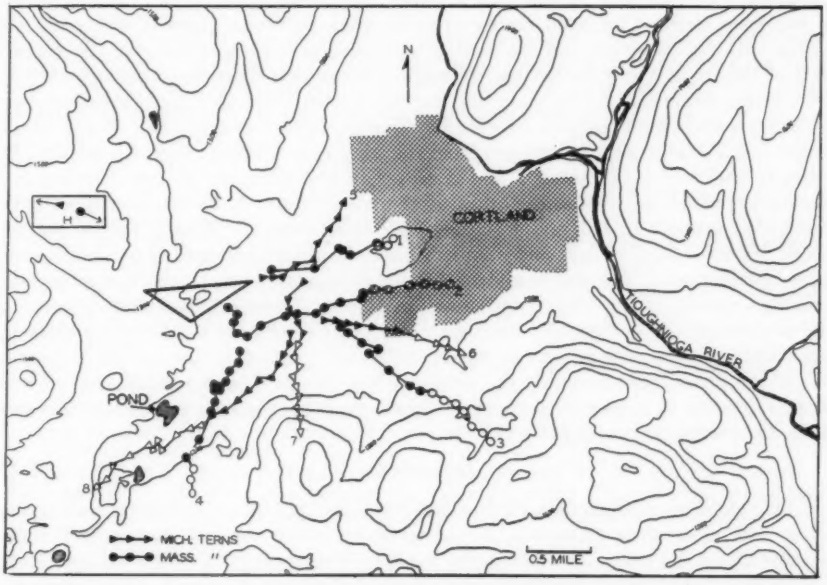

Figure 2. Sample flight paths of eight common terns. Solid circles and triangles stand for crossbearings, open circles and triangles for single bearings. Directions of the home colonies are shown by the arrows in the box (H) at the left. Positions of the observers are represented by the corners of the large triangle. Contour interval is 100 feet. See the text for a discussion.

The results are summarized in Figure 1, in which the points of the compass are "unrolled" on the horizontal axis; each bird is represented by a vertical rectangle. Only those birds are included that gave a distinct heading; the nine attracted to the pond and the seven lost while still circling less than 3000 feet from the release point have been omitted. Home direction is indicated by a capital "H" and a vertical line, the mean heading by an unmarked vertical line. The graph shows that the birds, from the Michigan colony at least, tended to head in a southeasterly direction. The average of the 19 Michigan terns was 139° (40–237°), while the average heading for the 6 Massachusetts terns was 93° (64–130°). In previous experiments

with terns from coastal colonies the average initial headings were 140°, 142°, 142°, and 149° when the home directions were 115°, 115°, 44°, and 211°, respectively. The Michigan terns released at Cortland thus showed nearly the same average heading as the coastal terns studied in previous seasons. The more easterly headings of the Massachusetts terns released at Cortland have no obvious explanation, but there were too few birds involved to render the difference between the two groups significant. While the individual headings varied more widely at Cortland than in our previous experiments, the distribution is by no means random, nor do the average headings differ significantly from our previous results.

Figure 2 is a selection of eight sample flight paths plotted from the simultaneous cross bearings and superimposed on a topographic map of the area. The portions of the lines marked by open circles and triangles represent parts of the path for which only one observer had the bird in view and are extrapolations of the earlier part of the flight (solid circles and triangles) for which there were cross bearings. For clarity, some of the first cross bearings from each flight path have been omitted. It is from such flight paths as these that Figure 1 was constructed. Birds numbered 5, 6, 7, and 8 (triangles) are from the Michigan colony, which lies about 335 miles to the west. Numbers 5 and 8 represent the extremes of the distribution; numbers 6 and 7, as well as the other fifteen from Figure 1, lie between these two. Similarly numbers 1, 2, 3, and 4 (circles) are terns from the Massachusetts colony, about 290 miles to the ESE, and numbers 1 and 4 are the extremes of the Massachusetts distribution. This figure shows that the topography had no obvious influence, although certain of the birds (not shown in Figure 2) were attracted to a pond to the southwest. For example, note that number 7 flew over a hill which rises to over 300 feet above the release point while number 2 flew over the city of Cortland. While the birds could certainly see terrain which lies beyond the boundaries of this map, reference to a smaller scale map shows no topographical features which explain the southeast headings.

Discussion

This experiment demonstrates that the tendency to fly in a southeasterly direction when first released in unknown, inland territory is not confined to common terns nesting along the coast; it was equally evident in terns from a colony in the Great Lakes area even though for these birds home lay almost due west. The same directional tendency has thus been observed when the home direction was northeast, east-southeast, south, southwest and west. Austin (1953), who has analyzed the migration route of this species on the basis of banding returns, has shown that both the Great Lakes and New England tern populations have a pronounced southeast component in the fall migration route. But whether this is relevant in the case of birds removed from their nesting grounds during the breeding season is open to question.

Arnould-Taylor and Malewski (1955) have recently suggested that topographic cues have been responsible for most of the results obtained by observing initial headings of homing birds. That topography may be the dominating influence under certain conditions has been firmly established; for example, Griffin (1952) reported that in a series of airplane observations of homing pigeons, certain of the birds followed roads, railroads, and lake shores. We have pointed out that in these experiments with common terns, local bodies of water attract many birds; but,

when precautions are taken to avoid release points with ponds in the vicinity, the terns tend to fly approximately southeast. Because it is so difficult to explain such observations on the basis of topographic cues, it is perhaps too soon to abandon all thought that birds employ some more refined method of navigation.

Summary

1. Our observations of the initial flight directions of common terns released in unfamiliar territory have been extended to include birds from the Great Lakes region as well as from the New England coast. When terns from both populations were released on the same day at Cortland, New York, both groups showed a tendency to head approximately southeast.

2. The first mile or two of the terns' flight paths were plotted on a topographic map of the area. Aside from the fact that a small pond attracted some of the birds, topography did not offer any apparent explanation of their headings, and a few persisted on a southeasterly course over moderately high hills.

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MODIFICATION OF X-RAY INJURY TO HYDRA LITTORALIS [1] BY POST-IRRADIATION TREATMENT WITH MAGNESIUM SULFATE AND GLUTATHIONE [2]

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Very few studies have been reported on the damaging effects of ionizing radiations on *Hydra*. Zawarsin (1929), Strelin (1929) and Evlakhova (1946), however, studied the effect of sublethal doses of x-rays and found that inhibition of budding and regeneration varied with the dose of radiation used. Daniel and Park (1951, 1953) reported a toxic effect of x-ray-treated media on *Hydra* tentacles and (1954) direct x-ray damage leading to death in 24 hours.

A number of investigators (Barron *et al.*, 1949; Bellack and Krebs, 1951; Chapman and Cronkite, 1950; Chapman *et al.*, 1950; Patt *et al.*, 1950; Bacq, 1951; Cronkite *et al.*, 1951) demonstrated that glutathione modifies some of the biological effects of ionizing radiations. In general, protection resulted only if the glutathione was given before irradiation. Similarly, in most cases cysteine has to be present at the time of irradiation in order to exert a protective effect (see Patt, 1953). Barron and co-workers, however, found that when glutathione was added to aqueous solutions of succinoxidase after irradiation, the enzyme was partially reactivated. Patt *et al.* (1952) reported protection to mammalian thymocytes when cysteine was added immediately after irradiation.

Daniel and Park (1954) showed that when hydras given 25,000 r were placed immediately in a dilute solution of salts containing either $MgSO_4$ or $MgCl_2$, about twice as many survived 24 hours as were living in the same salt solution without Mg^{++}. In view of this result, and of the few cases reporting modification of x-ray damage by post-irradiation treatment with sulfhydryl compounds, the present studies were made on the effects of continuous post-irradiation treatment with $MgSO_4$ plus glutathione on survival and on budding of hydras.

Materials and Methods

The hydras used in the present studies were from a clone culture grown in the laboratory at a room temperature of 25° ± 1.5° C. The cultures were kept in a standard salt solution of 1.7×10^{-3} *M* NaCl, 5.4×10^{-5} *M* KCl and 3.3×10^{-4} *M* $CaCl_2$ in double-distilled water (the second distillation being from glass). This solution contained the same salts and in approximately the same concentration as

[1] Kindly identified by Dr. Libbie H. Hyman, American Museum of Natural History, New York, N. Y.

[2] These data are from a thesis submitted to the Graduate Council of the George Washington University by Helen D. Park in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

that used by Daniel and Park (1954), and will hereafter be referred to as "standard saline." The hydras were fed newly hatched brine shrimp daily and were washed and changed to fresh standard saline one hour after each feeding. They were transferred to clean dishes once a week. Under these conditions the hydras reproduced asexually by budding.

For all experiments, hydras of equal size, without buds, were selected from the stock cultures before the daily feeding and washed in standard saline before treatment. The hydras were irradiated in a Pyrex glass dish containing 50 ml. of standard saline which gave a solution depth of 17 mm. The hydras immediately were washed with standard saline, then within 10 minutes were placed in the solutions to be studied. Except while the hydras were under observation the dishes were kept in moist chambers.

Irradiation factors were 50 kv constant potential, 50 ma beryllium window tube; 2700 r per minute. Dose determinations were made by the method of Andrews and Shore (1950). An aluminum plate 0.020 inch thick served as an x-ray filter and as a dish cover. Water cooling of the irradiation dish kept the temperature of the contents within 2° C. of the temperature of the laboratory.

Results

Survival experiments

In order to compare the effect of glutathione with that of $MgSO_4$, equal numbers of hydras exposed to 25,000 r were placed in (1) standard saline; and (2) 5.0×10^{-4} *M* $MgSO_4$, (3) 1.0×10^{-5} *M* glutathione, and (4) 5.0×10^{-4} *M* $MgSO_4$ plus 1.0×10^{-5} *M* glutathione, each in standard saline. Non-irradiated controls were also treated with the four solutions. The $MgSO_4$ concentration was within the range previously found by Daniel and Park to protect hydras exposed to 25,000 r. Within this range the protective effect of $MgSO_4$ was not a function of the ionic strength of the solutions. The concentration of glutathione had previously been shown to modify a toxic effect of irradiated water on hydra tentacles. The animals were left in their respective solutions 24 hours, at which time the survivors were counted. Five complete experiments, each consisting of 10 animals per group, were carried out in a period of 30 days.

Table I

Effect of post-irradiation exposure to $MgSO_4$ and glutathione on survival of hydras after 25,000 r. Fifty hydras in each treatment group

Treatment	Number alive after 24 hours	
	Irradiated	Non-irradiated
Standard saline	12±2	50
$5.0 \times 10^{-4}M$ $MgSO_4$ in standard saline	30±4	50
$1.0 \times 10^{-5}M$ glutathione in standard saline	18±4	50
$5.0 \times 10^{-4}M$ $MgSO_4$ + 1.0×10^{-5} *M* glutathione in standard saline	21±4	50

Standard error estimated from variation among 5 experiments.

As shown in Table I none of the non-irradiated hydras died. All of the irradiated groups showed by chi square test significantly [3] fewer survivors than their controls. The only statistically significant differences among the irradiated groups were between the hydras in $MgSO_4$ and those in saline, and between those in $MgSO_4$ and those in glutathione. There is not sufficient statistical evidence to assert definitely that either glutathione or the combined treatment had a protective effect against the radiation. The present results confirm the conclusion of Daniel and Park that $MgSO_4$ had a specific protective effect against the radiation.

Budding experiments

Hydras reproduce asexually by the formation of buds which constrict from the parent as adult hydras. The process involves increase in the mass of protoplasm, cell division and differentiation. In the stock cultures maintained in this laboratory, the development of a bud, from the time it is first recognizable until its separation from the parent, takes from two to four days.

The effects of continuous post-irradiation exposure to $MgSO_4$ and glutathione on budding were studied using 4500 r, a dose the author had previously found to be approximately one-third that necessary to inhibit bud production completely for 10 days. Forty irradiated and 40 non-irradiated hydras in groups of 10 were put in the standard saline, $MgSO_4$ saline, glutathione saline and $MgSO_4$ + glutathione saline solutions previously described. Beginning on the first day after irradiation, the hydras were fed daily. All descendents derived from the original 10 hydras in each group were kept with the parents. Each day for 11 days adults and attached buds were counted as separate individuals and all were transferred to fresh solutions in clean dishes. Five experiments, each including all of the treatment groups, were carried out at intervals over a period of two months.

For all analyses [4] of the data, statistical significance or the lack thereof was determined by comparing an average effect over the five replicate experiments with the variation of this effect among the five experiments.

Figure 1 shows, from days zero through eleven, the average number of adults plus buds present per experiment in each treatment group. Among the non-irradiated hydras, those in $MgSO_4$ and $MgSO_4$ + glutathione produced significantly greater numbers of descendents by the end of 11 days than those in standard saline or glutathione. Since neither of the other two intergroup differences among non-irradiated hydras was significant, it seems probable that during the combined exposure it was $MgSO_4$ which caused the increase in budding.

Comparison of the groups in saline alone shows that 4500 r depressed significantly the budding rate. The data for the irradiated hydras in standard saline suggest that the normal budding rate was regained by day nine, but since a parabola does not fit the points better than a straight line, the break in the curve may be fortuitous.

The budding rate of the irradiated hydras in $MgSO_4$ was not significantly greater than that of their irradiated controls. On the other hand, the irradiated hydras in glutathione produced buds at a significantly greater rate than the irradiated

[3] The .05 level of probability was used throughout the present work.

[4] The author wishes to thank Mr. Jerome Cornfield of the National Institutes of Health for his help in analyzing the data statistically.

controls. The irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated controls or the irradiated hydras in $MgSO_4$ or the irradiated hydras in glutathione, and at the same rate as the non-irradiated hydras in standard saline. It can be concluded, therefore, that under the conditions of these experiments, continuous post-irradiation exposure to $MgSO_4$ + glutathione restored the budding rate of irradiated hydras to that of non-irradiated hydras in standard saline.

In addition to showing the budding rates of all the groups, Figure 1 shows, on the

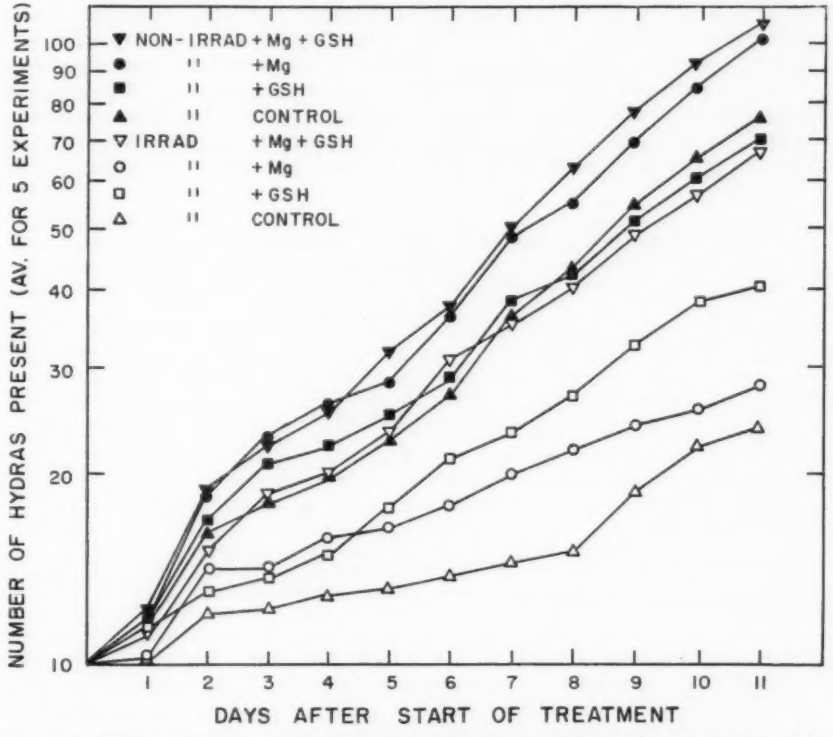

FIGURE 1. Effect of post-irradiation treatment with $MgSO_4$ and glutathione on budding of hydras after 4500 r. Treatment solutions were made in standard saline.

average, the time at which all 10 of the hydras in each group initiated their first buds (*i.e.*, when 20 adults and buds were present in each group). The data for the individual experiments show that all 50 of the non-irradiated hydras in standard saline initiated their first buds by day six. The total number of irradiated hydras in each treatment group that had initiated their first buds by the time all first buds appeared in the non-irradiated standard saline group was: standard saline 17, $MgSO_4$ 39, glutathione 35, and $MgSO_4$ + glutathione 47. It can be concluded that one of the effects of the radiation was to delay the time of appearance of first buds.

Magnesium sulfate, glutathione, and $MgSO_4$ + glutathione reduced the severity of the radiation effect, but only the combined treatment shortened the time of first bud initiation to that of the non-irradiated, saline controls.

Since the irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated hydras in $MgSO_4$ alone or glutathione alone, the effect of concentration of the two agents on budding after irradiation was studied in order to ascertain whether the greater effect produced by the two agents together is valid when related to optimal effects of each when used separately. Accordingly, hydras were irradiated with 4500 r and placed in groups of five in solutions in which both $MgSO_4$ and glutathione concentrations were varied from ½ to 16 times those used in the preceding experiments. The hydras were counted on the eleventh day after irradiation.

Table II shows the mean number of hydras present on the eleventh day in each treatment group. The results presented in column 1 show that up to a concentration of 8.0×10^{-3} mole per liter, $MgSO_4$ did not modify the inhibiting effect of 4500 r of x-rays. The results shown in line 1 of the table indicate that glutathione in

TABLE II

Effect of concentration of $MgSO_4$ and glutathione on budding of hydras after 4500 r. Mean number of individuals present on 11th day post-irradiation per 5 hydras treated

Moles per liter of $MgSO_4$	Moles per liter of glutathione						
	0.0	5.0×10^{-6}	1.0×10^{-5}	2.0×10^{-5}	4.0×10^{-5}	8.0×10^{-5}	1.6×10^{-4}
0.0	5.0(5)	6.5(4)	9.0(4)	6.8(4)	10.0(4)	5.0(3)	0.3(3)
2.5×10^{-4}	5.5(4)	13.0(3)	11.3(3)	12.3(3)	11.0(3)	6.0(3)	2.0(3)
5.0×10^{-4}	5.0(5)	9.3(3)	13.0(2)	17.3(3)	19.0(3)	8.3(3)	4.0(3)
1.0×10^{-3}	5.2(4)	9.3(3)	12.0(3)	10.3(3)	13.0(3)		1.7(3)
2.0×10^{-3}	5.4(5)		12.0(3)		22.0(4)	5.0(3)	
4.0×10^{-3}	5.7(3)		10.3(3)		14.0(1)	10.0(3)	1.0(1)
8.0×10^{-3}	5.0(3)		6.0(2)			6.0(2)	4.0(2)

Figures in parentheses = number of groups of hydras treated. The mean number of individuals present on the 11th day in 5 groups of 5 non-irradiated hydras in standard saline was 12.0. No concentration tests were run on non-irradiated hydras.

concentrations between 5.0×10^{-6} and 4.0×10^{-5} mole per liter reduced the inhibitory effect of the radiation on bud production; 1.6×10^{-4} *M* glutathione was highly toxic. Radiation was probably not a factor in this toxicity as five non-irradiated hydras placed in this solution were dead five days later. The data show that the optimal concentrations of the two agents when supplied together were in the range of 2.0×10^{-5} to 4.0×10^{-5} *M* glutathione and 5.0×10^{-4} to 2.0×10^{-3} *M* $MgSO_4$. In addition they show that combined treatment within these ranges resulted in greater bud production than at optimal concentrations of either agent used separately. Furthermore, the amount of budding that took place during exposure to optimal concentrations of both agents together was as great as that of the non-irradiated hydras in standard saline.

DISCUSSION

At first glance the effects of $MgSO_4$, glutathione and combined treatment, after the two radiation exposures employed, appear to be anomalous. Since the two sets

of results are expressed in different units—number surviving out of total number treated, and rate of increase in numbers of hydras present—they cannot be compared statistically. Taking the apparent discrepancies at face value, however, it seems reasonable that a particular agent might be more effective against a mild cellular damage which would partially inhibit budding than against a more drastic injury leading to death in 24 hours, or that another agent might be more effective in keeping an animal alive for 24 hours than in maintaining it in a reproductive state for a period of 10 days.

The mechanism of the stimulating action of $MgSO_4$ on budding of non-irradiated hydras is not known. However, this effect is perhaps not surprising in view of the fact that $MgSO_4$ has been shown to affect growth in many organisms as widely separated phylogenetically as bacteria (Webb, 1953), protozoa (Mast and Pace, 1939), and mammals (Kruse *et al.*, 1932). Since the addition of increasing amounts of $MgSO_4$ did not increase the amount of budding of irradiated hydras, we may conclude that lack of $MgSO_4$ was not the factor which limited budding after irradiation.

Mechanisms of radiation protection have been considered in reviews by Ord and Stocken (1953) and by Patt (1953). One of the theories of protection by sulfhydryl compounds is that there is a competition by —SH groups for free radicals formed from water in an irradiated solution. Since, in the study reported here, the hydras were not irradiated in the presence of glutathione, and were washed immediately after irradiation and at least ten minutes elapsed between the end of irradiation and beginning treatment with glutathione, the effect on budding would seem to have been due to some mechanism other than a competition of —SH groups for free radicals within or at the surface of the hydra cells.

It is not known whether hydras need an external source of glutathione for budding. If they do, it is possible that the reason glutathione did not stimulate the budding of the non-irradiated hydras was because they were already getting a sufficient amount for budding in their normal intake of food. If hydras do not need an external source of glutathione for budding, stimulation would not occur on the addition of glutathione to the medium.

It was not practicable to determine the amount of food eaten by any of the hydras. However, if the irradiated hydras ate less food than the controls, the rate of budding would be reduced from that of the controls. The effect of glutathione in increasing the budding rate of irradiated hydras might thus have been due to the fact that this agent stimulates mouth opening (Loomis, 1955) which in turn might permit the hydras to consume more food. A second possibility is that the requirement of irradiated hydras for glutathione or some part of the molecule may be greater than that of non-irradiated hydras, *e.g.*, because of the reconstitution of injured regions. Thus the requirement might not be met even with normal food intake, causing a decreased budding rate; addition of glutathione to the medium might satisfy the greater requirement and increase the budding rate over that of the irradiated controls.

The fact that $MgSO_4$ stimulated budding of non-irradiated hydras that were presumably getting an adequate amount of glutathione through their normal intake of food, and the fact that none of the concentrations of $MgSO_4$ used after irradiation had a significantly stimulating effect unless added glutathione was present,

suggest the possibility that in all hydras, stimulation of budding by $MgSO_4$ depends on the presence of an adequate level of glutathione or sulfhydryl in the hydra tissues.

Summary

1. Hydras were left for 24 hours in solutions of $MgSO_4$, glutathione and $MgSO_4$ + glutathione after exposure to 25,000 r x-rays. Only the hydras in $MgSO_4$ alone were significantly protected against the effects of the radiation.

2. Hydras were exposed continuously to $MgSO_4$, glutathione, and $MgSO_4$ + glutathione solutions after 4500 r. The rates of budding in each solution were determined. It was found that:

(a) Forty-five hundred r inhibited the budding of hydras significantly.
(b) Magnesium sulfate and $MgSO_4$ + glutathione stimulated budding of non-irradiated hydras while glutathione alone did not.
(c) Magnesium sulfate alone did not significantly modify the inhibitory effect of the radiation on budding.
(d) Glutathione alone partially reversed the inhibitory effects of the x-rays.
(e) Within a fairly wide range of concentrations of $MgSO_4$ and glutathione, the two agents together restored the budding rate of irradiated hydras to that of the non-irradiated animals in standard saline.

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THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, AZYGIA SEBAGO WARD, 1910[1]

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The genus *Azygia* was erected by Looss (1899) to contain *Fasciola tereticollis* Rudolphi, 1802 (= *Fasciola lucii* Mueller, 1776, renamed). The worms were from the stomach of *Esox lucius*. According to Dawes (1946), this species, *Azygia lucii* (Mueller, 1776) Lühe, 1909, infects a number of different salmonid fishes, and other species of *Azygia* described from Europe are identical with it. The species has been reported in North America as *Distoma tereticolle* by Leidy (1851) from *Esox reticulatus;* by Stafford (1904) from *Esox lucius, Lota maculosa* and *Ameiurus nigricans;* and as *Azygia lucii* by Cooper (1915) from *Lucius lucius* (= *Esox lucius*), *Lucius masquinongy* (= *Esox masquinongy*), *Lioperca* sp., and immature specimens presumably of the same species were found in *Salvelinus namaycush* and *Micropterus dolomieu.*

Meanwhile, other species of *Azygia* were described in the United States and Canada. Leidy (1851) described *Distoma longum* on the basis of six specimens from the stomach of *Esox estor* Lesueur, 1818 (the American pike), collected near Cleveland, Ohio, and received from Professor Spencer F. Baird. The worms measured 30 to 76 mm. (3 inches) in length and as much as 1.6 mm. in breadth; the maximum diameter of the oral sucker was 1.27 mm. and of the acetabulum 1.06 mm. Measurements given by Leidy for specimens from the stomach of *E. reticulatus,* which he identified as *Distoma tereticolle* Rudolphi, were: length up to 17 mm.; width, 1.06 mm.; oral sucker, 0.52 mm.; and acetabulum 0.7 mm. There is some confusion here since Manter (1926) (p. 66) reported, "Leidy's *Dist. tereticolle* (from *Esox reticulatus*) also was compared with them (specimens of *D. longum* from the Leidy and Cooper collections), and in the single specimen available in the Leidy collection, the oral sucker, contrary to Leidy's description, was found to be slightly larger than the acetabulum." Stafford (1904) erected the genus *Megadistomum* to contain specimens from *Esox masquinongy* which he regarded as identical with *Distoma longum* of Leidy and distinct from *Azygia tereticollis.* Specimens of *Megadistomum longum* (Leidy, 1851) measured up to 5 inches in length when fully extended and up to 3 mm. in breadth, whereas those identified as *A. tereticollis* measured 12 mm. in length and 1 mm. in width. Stafford reported that the largest specimens of *A. tereticollis* were smaller than immature specimens of *M. longum.* Furthermore, he described worms from the stomachs of *Lota maculosa* and of *Stizostedion vitreum* as members of a new genus and species, *Mimodistomum angusticaudum.*

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Marshall and Gilbert (1905) described *Azygia loossi* from the large-mouth bass, *Micropterus salmoides;* the pike, *Lucius lucius;* and the bowfin, *Amia calva.* The worms contained only a few eggs and obviously were not fully mature. They measured 5 to 7 mm. in length, 0.5 mm. in width; the acetabulum was near the middle and the gonads in the caudal one-sixth of the body.

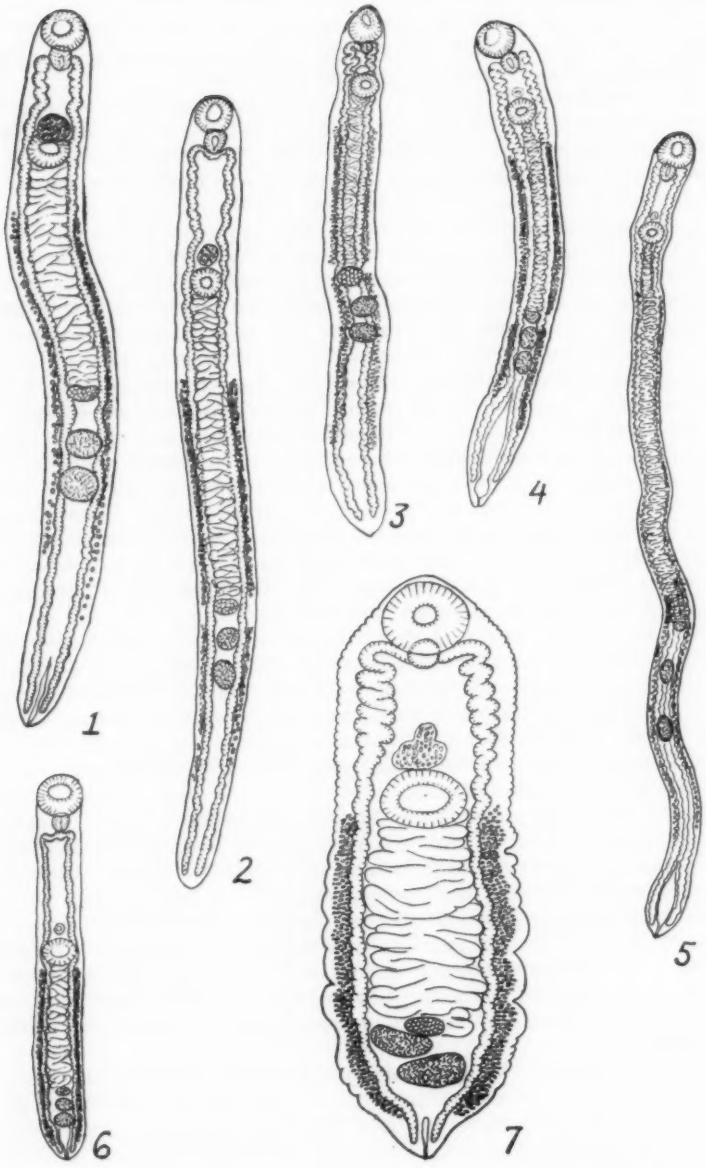
Ward (1910) described *Azygia sebago* from *Salmo sebago* taken at Lake Sebago, Maine. All of seven fishes examined were infected; the worms measured up to 10 mm. in length and from 0.7 to 1.0 mm. in width. From the magnification given, the figured specimen was about 6 mm. long and 0.8 mm. wide. The average diameter of the oral sucker was given as 0.68 mm. and the acetabulum was "distinctly smaller." Specimens presumed to belong to the same species were found in other fishes of Lake Sebago. Two worms were removed from the stomach of a single specimen of *Perca flavescens* and measurements were given for one of them. It was 4.08 mm. long, 0.77 mm. wide; the oral sucker measured 0.51 by 0.57 mm. and the acetabulum 0.35 by 0.40 mm. Four of nine eels, *Anguilla chrysypa* (= *A. rostrata*) were infected with an average of three worms per fish. No descriptive data were given, so presumably they conformed to the specific diagnosis. Eleven of twelve young *Esox reticulatus* were heavily infected; as many as 80 worms were found in a single host. These parasites were more slender and measured 10 to 18 mm. in length. It may be doubted whether they are conspecific with the shorter, more robust worms from the other hosts. Ward reported that smelt, *Osmerus mordax,* were eaten by the larger fishes; the parasites were found also in the stomachs of smelt, although in this host the worms were usually smaller and sexually immature. He noted that the specimens identified by Stafford (1904) as *Azygia tereticolle* are smaller (12 mm. long and 1 mm. wide) than the European species and expressed the belief that they may have been *A. sebago.*

Goldberger (1911) recognized *A. loossi* as a valid species and did not mention *A. sebago,* as Ward's account was probably not available when he wrote his paper. He reported on specimens collected from *Amia calva* taken in Indiana lakes; certain of the worms were identified as *A. lucii,* and others were described as members of two new species, *Azygia bulbosa* and *Azygia acuminata.* Also, he described worms from the stomach of the rock bass, *Ambloplites ruprestris,* as members of a new genus and species, *Hassallius hassalli.*

Odhner (1911) erected the family Azygiidae to contain *Azygia, Otodistomum, Leuceruthrus,* and *Ptychogonimus.* He stated that in the genus *Azygia,* measurements of eggs and extent of vitellaria have little value for specific determination. He declared that *Megadistomum longum* (Leidy, 1851) Stafford, 1904 and *Mimodistomum angusticaudum* Stafford, 1904 are members of the genus *Azygia* and the two generic names were relegated to synonymy. He suggested the probable identity of *A. tereticollis* of America with *A. lucii* of Europe. He criticized Goldberger's work, suppressed *Hassallius* as a synonym of *Azygia,* and expressed the belief that *A. angusticauda, A. loossi, A. acuminata,* and *A. bulbosa* are members of a single species.

As noted, Cooper (1915) described worms which he identified as *A. lucii* from the pike, *Lucius lucius;* the muskellunge, *Lucius masquinongy; Lucioperca* sp.; and immature specimens were recovered from *Salvelinus namaycush* and *Micropterus dolomieu.* He stated that all the worms from the muskellunge are identical with Stafford's *Megadistomum longum* (Leidy) and the smallest one with eggs was 8

PLATE I



mm. long. Cooper noted the variable size of worms at the time of egg production. The smallest gravid specimen from the pike was 6 mm. long, but another from the pike, 14 mm. long, was less mature than the one 6 mm. long; others 6 to 14 mm. in length were fully gravid. All the worms from the trout, *S. namaycush*, and the black bass, *M. dolomieu*, including the largest one, 11 mm. long, were immature. Other young and immature specimens from the stomach of *Perca flavescens* were regarded as possible members of this species. Worms from the pickerel (not named) resembled *A. angusticaudum* (Stafford, 1904) but were too contracted to permit positive identification, and others from the pike had a large, globose excretory vesicle, described by Goldberger as characteristic of *A. bulbosa*, but Cooper stated that the shape of the excretory vesicle as well as the length, extent, and "breaking" of the vitellaria are so variable as to be of little use in the delineation of species. Cooper recognized the validity of *A. acuminata*, since 9 specimens from the stomach of *Amia calva* agreed substantially with Goldberger's description of this species.

Ward (1918) stated (p. 392), "Despite many records of its occurrence, the common European *A. lucii* (= *A. tereticolle*) has not been found in North America. Several species peculiar to this continent occur in *Amia calva, Micropterus salmoides* and *dolomieu, Esox lucius* and *reticulatus, Ambloplites ruprestris, Salvelinus namaycush, Lioperca, Lota lota,* and *Salmo sebago.*"

Manter (1926) gave a systematic review of the family Azygiidae; he agreed with Ward in regarding the American specimens as specifically distinct from those of Europe but admitted (p. 57) that "*Azygia* is the only genus of the family showing taxonomic confusion in its species." Accepting the statements of Odhner and Ward, he distinguished *Azygia longa* from *A. lucii* on the extent of the vitellaria, which in the European species are reported not to extend behind the testes, and on the shape of the pharynx, which in *A. lucii* is reportedly cylindrical and twice as long as wide. After detailed study and tabular comparison of morphological features, Manter recognized only three species of *Azygia* in North America, *viz., A. longa* (Leidy, 1851), *A. angusticauda* (Stafford, 1904), and *A. acuminata* Goldberger, 1911. Manter confirmed the suspicion of Odhner (1911) that *Azygia loossi* is identical with *Mimodistomum angusticaudum* Stafford, 1904. As syno-

PLATE 1

FIGURE 1. *Azygia lucii*, from *Esox lucius;* specimen collected and identified by Prof. M. Braun, Königsberg, 7 July 1902; 23 mm. long, ventral view; U. S. National Museum, Helminthological Collection No. 3359.

FIGURE 2. *Azygia lucii*, from *Amia calva;* 20 mm. long, ventral view, (Ward Collection) U. S. N. M., Helminth. Coll. No. 51,403.

FIGURE 3. *Azygia longa*, from *Esox reticulatus*, identified by Albert Hassall; 12.4 mm. long; U. S. N. M., Helm. Coll. No. 49.

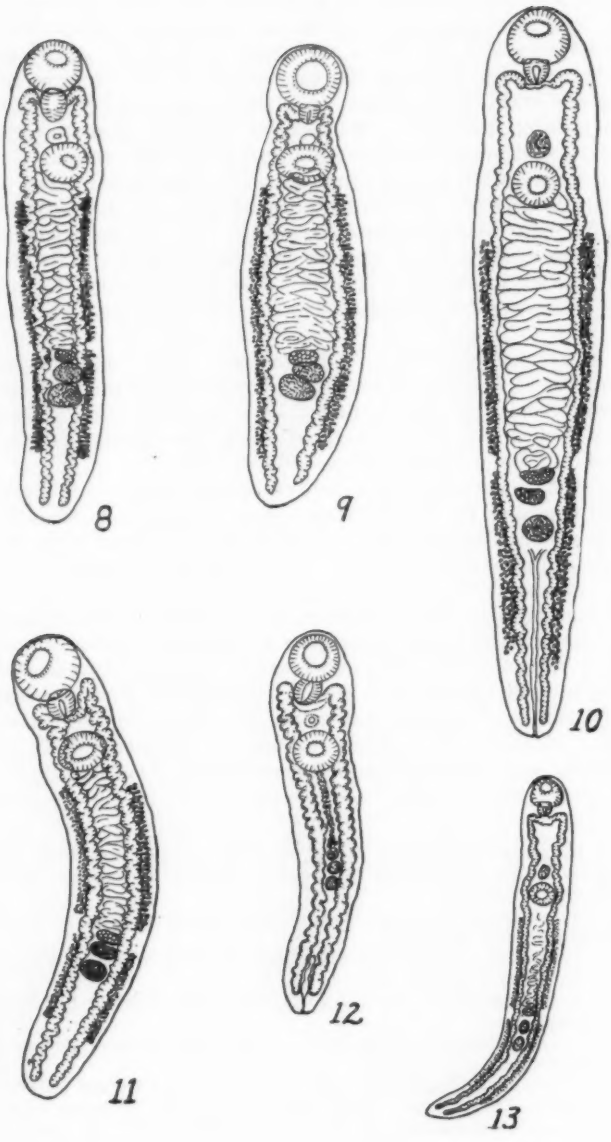
FIGURE 4. *Azygia longa*, from *Esox niger;* 5.2 mm. long, collected 1955 by Paul Krupa, southern New Hampshire.

FIGURE 5. *Azygia longa*, from *Esox niger;* 19 mm. long, collected 1955 by Paul Krupa, southern New Hampshire.

FIGURE 6. *Azygia angusticauda* (type of *Azygia loossi*, Marshall and Gilbert, 1905), from *Micropterus salmoides;* 4.88 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10,679.

FIGURE 7. *Azygia angusticauda*, from *Stizostedion vitreum;* 10.5 mm. long; ventral view, (Ward Collection) taken by H. W. Manter 4 April 1926, Rock River, Illinois; U. S. N. M., Helm. Coll. No. 51,402.

PLATE II



nyms of *A. longa* (Leidy), Manter listed: *Distomum longum* Leidy, 1851; *Distomum tereticolle* of Leidy, 1851; *Megadistomum longum* (Leidy) of Stafford, 1904; *Azygia tereticolle* of Stafford, 1904; *Azygia sebago* Ward, 1910; *Azygia bulbosa* Goldberger, 1911; *Hassallius hassalli* Goldberger, 1911; and *Azygia lucii* of Cooper, 1915. He discussed the problems of specific determination, noted the bundles of longitudinal muscles which traverse the parenchyma and quoted Leuckart's description of them, and stated that in such elongate and powerfully muscled trematodes, contractions not only alter the general shape of body but the form and relative position of internal organs. Concerning differences in size and sexual maturity, he observed that in the related species, *Otodistomum cestoides*, specimens increase six to seven times in size after attainment of sexual maturity. This fact was used to justify the inclusion in a single species, *A. longa*, of gravid specimens 3.9 mm. long which had been described as *A. bulbosa*, and others which measured up to 3 inches in length and had been described as *A. longum*. It is true that these specimens were from different host species and worms grow larger in larger hosts, but it is doubtful whether host influences can produce such extreme range in size within a single species. Manter's description of *A. longa* was based largely on worms which Ward had described as *A. sebago* and which Manter regarded as identical with *A. longa*. The specific features of *A. sebago* were not clearly defined; there is uncertainty concerning the species, since there is strong probability that material of more than one species was included in the specific diagnosis. According to Manter who studied the Ward collection (p. 64), "*A. sebago* averages about 6 to 8 mm. in length. Specimens were found as small as 1 mm. and no ova were present in forms 2.85 mm. long. . . . Of the other *Azygia* species, *A. bulbosa* Goldberger is most evidently identical with *A. sebago*. Type material of both species was studied. . . . The original type material of *Hassallius hassalli* was also examined for comparison. . . . In fact, after allowance is made for body contraction, this form can not be distinguished from the other common American forms as represented by *A. sebago* and *A. bulbosa*."

Van Cleave and Mueller (1934) remarked on the variability in fundamental characters, such as the anterior and posterior limits of the vitellaria and the position of the gonads, in the genus *Azygia*. They endorsed the action of Manter in reducing the number of species in North America and went even further in reducing *A. acuminata* to synonymy with *A. longa*. They noted that Manter had listed *A. bulbosa* as a synonym of *A. longa*, and since they regarded *A. acuminata* and *A.*

PLATE II

FIGURE 8. *Azygia sebago*, from *Perca flavescens*, Sebago Lake, Maine, 1907, 4.26 mm. long, ventral view, (Ward Collection); U. S. N. M., Helm. Coll. No. 51,401.

FIGURE 9. *Azygia acuminata*, from *Amia calva*, Indiana, type of Goldberger, 1911, 6.6 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10,500.

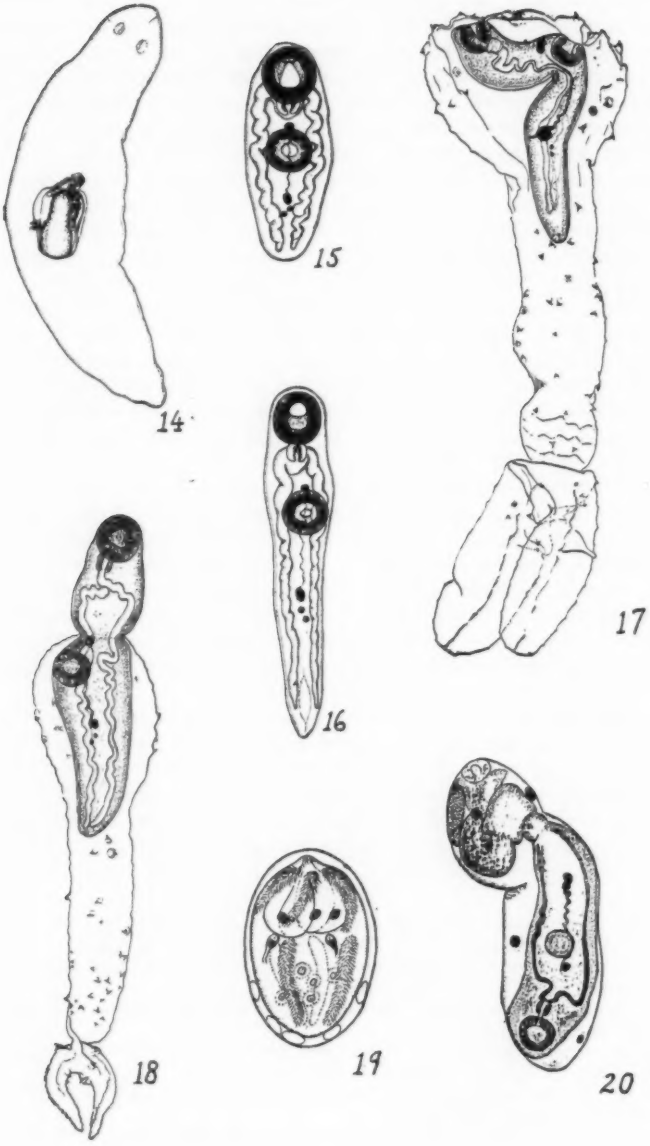
FIGURE 10. *Azygia sebago*, from *Anguilla rostrata*, Falmouth, Mass., 1955, flattened specimen, 12.5 mm. long, ventral view.

FIGURE 11. *Azygia bulbosa*, from *Amia calva*, Indiana, type of Goldberger, 1911, 8.6 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10502.

FIGURE 12. *Azygia sebago*, from *Anguilla rostrata*, Falmouth, Mass., 1954, immature specimen, 2.66 mm. long, ventral view.

FIGURE 13. *Azygia sebago*, from *Anguilla rostrata*, Falmouth, Mass., 1955, young specimen with 46 eggs in the initial one-half of the uterus, 5.3 mm. long, ventral view.

PLATE III



bulbosa as synonyms, *A. acuminata* should also become a synonym of *A. longa*. The reasoning is sound if the postulates are correct, which now appears doubtful. The specimen shown in their Figure 9 (5) which is referred to *A. angusticauda* and the one Figure 9 (7) referred to *A. longa* are so similar that they are probably conspecific and they may not belong to either *A. angusticauda* or *A. longa*. They closely resemble the worms from the eel, identified in this paper as *A. sebago*.

In other surveys of trematode parasites of fishes, *A. angusticauda* and *A. longa* have been reported in eastern North America but *A. longa* may not extend into the area of Lake Huron and northern Wisconsin and neither species has been found in the fishes of western Canada. Lyster (1939) reported *A. longa* from *Esox lucius* and *Anguilla rostrata* in Canada. The single worm from *E. lucius* is probably a young specimen of *A. longa*, but those from eels are very different. He stated that some of them could be assigned to *A. angusticauda* and specimen No. 1 in his table, which is 4.8 mm. wide at the acetabulum, is probably *A. angusticauda*. The others, which are the same length as the one from *E. lucius* but are twice as wide, are very similar to those from eels on Cape Cod. Miller (1940) reported *A. angusticauda* from *Stizostedion vitreum* and *Micropterus dolomieu* in the central St. Lawrence watershed. Miller (1941) restudied the collection of Stafford. He found a specimen from the muskellunge which he identified as *Megadistomum longum;* it was 18.5 mm. long, 1.2 mm. wide, and there are no eggs in the uterus. Another specimen, from *Lota maculosa* and identified as *A. tereticolle*, is 6.5 mm. long, 0.5 mm. wide, and contains eggs. If these worms belong to *A. longa*, as stated, it is difficult to explain the sexual maturity of the smaller individual. In the Stafford collection Miller found two mature and several juvenile specimens of *Mimodistomum angusticaudum*. One of the mature specimens, 7.25 mm. long and 1.65 mm. wide, shown in his Figure 13, is typical, with the acetabulum near the middle and the gonads in the posterior one-sixth of the body. This account of the original Stafford specimens definitely relegates *Azygia loossi* Marshall and Gilbert, 1905 to synonymy with *A. angusticauda* (Stafford, 1904). Choquette (1951) reported both *A. longa* and *A. angusticauda* from the muskellunge, *Esox m. masquinongy*, in the St. Lawrence watershed. Meanwhile, Bangham (1944) examined 1,330 fishes, representing 38 different species, from 40 different locations in northern Wisconsin. He did not find *A. longa*, but *A. angusticauda* was present in 12 species of fish. Bangham and Venard (1946) examined 676 fishes, belonging to 22 species, from Algonquin Park lakes. Worms from *Anguilla rostrata* were

PLATE III

FIGURE 14. *Dugesia tigrinum*, 7 mm. long, experimental infection, two juvenile *Azygia sebago* in the pharyngeal pockets.

FIGURE 15. *Azygia sebago*, 0.81 mm. long, natural infection, from pharyngeal cavity of *D. tigrinum*, juvenile worm found by J. Louis Bouchard.

FIGURE 16. *Azygia sebago*, juvenile worm from *D. tigrinum*, 1.17 mm. long, natural infection, specimen from J. Louis Bouchard.

FIGURE 17. *Azygia sebago*, cercaria, naturally emerged, a fixed and stained specimen.

FIGURE 18. *Azygia sebago*, cercaria, from a crushed snail, larva not entirely mature and only partially enclosed in the enlarged, basal end of the tail, furci shriveled, a fixed and stained specimen.

FIGURE 19. *Azygia sebago*, miracidium in egg, from sketches made of living larvae.

FIGURE 20. *Azygia sebago*, redia in which the pharynx is recognizable; the body of the cercaria is 0.8 mm. long, the furci 0.18 mm. long; fixed and stained specimen.

identified as *A. longa;* others from *Micropterus dolomieu, Perca flavescens* and *Lepomis gibbosus* were identified as *A. angusticauda.* Bangham and Adams (1954) did not find *Azygia* in the examination of 5456 fishes, belonging to 36 different species, taken in the Columbia, Fraser and other rivers of western Canada. In a survey of parasites from 1667 fishes, representing 53 species, from Lake Huron and Manitoulin Island, Bangham (1955) found *A. angusticauda* in the northern channel catfish, *Ictalurus l. lacustris.* This parasite obviously can infect a large number of species of fish.

Knowledge of the life-history of azygiid trematodes dates from the publication by Szidat (1932) on the developmental cycle of *Azygia lucii,* a common parasite in the stomachs of salmonid fishes, especially species of *Esox,* in Europe. Szidat found that the large, furcocercous, cystocercous larva, *Cercaria mirabilis* Braun from *Lymnaea palustris,* when fed to young pike, *Esox lucius,* developed in ten days into adult *Azygia lucii.* He recalled the statement of Looss (1894), that when small pike are eaten by larger ones, the azygiid parasites leave the stomach of the ingested fish and establish themselves on the stomach of the preditor; and stated (p. 501), "Überdies sind ältere Hechte keine Planktonfresser mehr, so dass für sie die Übertragung auf dem zuletzt geschilderten Wege den vorherrschenden Modus darstellen wird, und die jugendlichen Hechte demnach biologisch doch als Zwischen- oder Hilfswirte zu werten sind." Szidat reported that other small fishes also ingest the cercariae and may serve as transport hosts, but in these species the parasites do not develop to sexual maturity. He found juvenile *A. lucii* in the stomachs of small predacious fishes belonging to the genera *Perca, Lucioperca,* and *Gasterosteus.* Szidat described the cercaria-producing generation as a redia, which lacks a digestive tract but in which the pharynx persists as an organ for ingesting fragments of the digestive gland of the snail host and also as a birth pore. He traced the development of the cercariae and noted their resemblance to those of the strigeids and schistosomes. The cercariae are not encysted in the snail host. The body of the cercaria sits in a narrow depression at the anterior end of the flattened tail-stem. The cercariae mature in the haemocoele of the snail and emerge into the mantle cavity. In water, the proximal portion of the tail begins to swell and the body of the larva, anchored in the base of the depression by the tubule of the excretory system, is enveloped by the base of the tail and enclosed in it. Szidat also described *Cercaria splendens,* believed to represent a second species of *Azygia,* but the adult stage and final hosts were not discovered.

The achievement of Szidat in working out the life-cycle of *A. lucii* disclosed that the furcocercous, cystocercous larvae of the Mirabilis type, originally regarded by Leuckart as free-swimming sporocysts and shown by Braun (1891) to be cercariae when he described *Cercaria mirabilis,* are developmental stages of azygiid trematodes. The first member of the group was found by Wright in a fresh-water aquarium and described (1885) as a free-swimming sporocyst. Ward (1916) named the species *Cercaria wrighti* and described a second species, *Cercaria anchoroides,* collected in top and bottom tow every day from July 25 to August 5, 1893, in Lake St. Clair, Michigan. Subsequent investigators have reported other members of the Mirabilis group; sixteen species have been described, but some of them are identical. Several of the named species were described from immature stages, taken from crushed snails, and can not be identified with certainty. Others were described from free-swimming cercariae and the hosts are unknown. Certain

of them have proved to be larvae of species in the genus *Proterometra*, erected by Horsfall (1933) to contain *Cercaria macrostoma* Faust, 1918. Reviews of the cystocercous cercariae were published by Horsfall (1934), Smith (1936) and Dickerman (1946). Those with forked tails were designated as furcocystocercous by Le Zotte (1954) who showed that members of the family Bivesiculidae also have larvae of this type.

The second report on the life-cycle of azygiid trematodes was given by Stunkard (1950). Larval distomes had been referred to him for identification in the winter of 1949–1950 by Mr. J. Louis Bouchard, then a graduate student at the University of Oklahoma. The worms had been found in planarians, *Dugesia tigrinum*, received from the Marine Biological Laboratory, Woods Hole, Massachusetts. The structure of the larvae indicated that they were azygiids and study of the life-cycle was begun at the Marine Biological Laboratory in the summer of 1950. Records of the Supply Department of the M. B. L. showed that the planarians sent to the University of Oklahoma had been collected in Morse's Pond in Falmouth. Thirty-eight *D. tigrinum* were collected there on July 10, 1950 and a larval trematode, identical with the specimens sent by Mr. Bouchard, was found in the pharyngeal pockets of two of them. Eight additional worms of natural infection were found in 120 *D. tigrinum* examined. To discover the first intermediate host, different species of mollusks were collected from Morse's Pond and isolated. Furcocercous cercariae of the azygiid type emerged from nine of 246 *Amnicola limosa*. Four planarians, examined under the microscope and known to be uninfected, were placed in a finger-bowl with three specimens of *A. limosa* which were shedding these cercariae. After six days exposure, one to four larvae were found in the pharyngeal cavities of each of the planarians. These larvae were identical with those sent by Mr. Bouchard. The tails, in the bases of which the bodies of the cercariae formerly were enclosed, had completely disappeared. Other planarians were subsequently placed in dishes with infected *A. limosa* and larvae found in their pharyngeal pockets. The larvae may persist for several weeks in *D. tigrinum*, but they do not encyst or grow and it is apparent that the planarians serve merely as paratenic or transport hosts. Attempts to feed the cercariae to goldfish and small perch were not successful; the fish would not take the larvae and when introduced into their mouths, the cercariae were expelled. Planarians infected with cercariae were fed, but the results were uncertain and the short period in which the work could be conducted, led to no further information at that time. It was clear that the larvae belonged to a species of *Azygia*, but specific determination could not be established.

Sillman (1953a) reported that in the vicinity of Ann Arbor, Michigan, the mud pickerel, *Esox vermiculatus*, and the bowfin, *Amia calva*, harbor *Azygia longa*. Eggs of the trematode, containing mature miracidia, were fed to both wild and laboratory-raised *Amnicola limosa*. Cercariae producing rediae were found after 21 days and cercariae emerged 42 days after infection. Cercariae fed to *Esox vermiculatus* developed in 20–30 days into egg-bearing worms. Two of 13,500 *Amnicola limosa* were found naturally infected with cercariae which appeared identical with those in experimentally infected snails.

In a thesis submitted for the Ph.D. degree at the University of Michigan, Sillman (1953b) gave further information. He stated that two species of *Azygia* are present in the Ann Arbor area. One species, which he identified as *A. longa*, occurs in both *Esox vermiculatus* and *Amia calva*. The other species, which he iden-

tified as *A. acuminata*, was found only in *Amia calva*. Worms assigned to *A. longa* were somewhat longer, more slender and the suckers were slightly smaller than those of *A. acuminata*, but the measurements of worms and organs overlapped. According to Sillman, the collecting ducts of the excretory system branch from the vesicle behind the testes in *A. longa* and between the testes in *A. acuminata*. Although there was much variation, the average size of eggs in *A. longa* was 55 by 31 microns whereas that of *A. acuminata* was 69 by 38 microns. Furthermore, specimens of *Amnicola limosa* did not become infected when fed eggs of *A. acuminata*.

Investigation of the life-cycle and development of *Azygia* has been continued at the Marine Biological Laboratory, Woods Hole, Mass., during the summer months since 1950. An abstract of the results was presented (Stunkard, 1955). Infected snails were found each year and the morphology of the young distome, especially the details of the excretory system, was studied. Hundreds of fishes, including *Esox niger, Perca flavescens, Morone americanus, Micropterus salmoides, Micropterus dolomieu*, and others, were examined in the attempt to find the sexually mature stage of the parasite. The first to be discovered, a small, immature specimen of *Azygia* (Fig. 12) was found in the stomach of an eel, *Anguilla rostrata*, late in the summer of 1954. During the summer of 1955, 42 eels were examined; 10 of them were infected and many fully mature worms were collected. Continued examination of other fishes, especially the pickerel, *Esox niger*, from the same ponds where the infected eels were taken, has not disclosed infection by members of the genus *Azygia*, and it appears that the eel is the natural and possibly the only host for the species in the Woods Hole region. The larger ponds in the area are under the control of the Division of Fisheries and Game, Bureau of Wildlife Research and Management of the State of Massachusetts, and many of them have been stocked with game fishes from time to time. Through the kind cooperation of Mr. Russell Cookingham, a large number of fishes, belonging to various species, were provided during the summer of 1955, when certain of these ponds were inspected to determine their productivity.

Specific determination of the parasites from the eel has proved difficult. Descriptions are wholly unsatisfactory and accordingly, specimens of *Azygia* in the U. S. National Museum were borrowed through the kindness of Dr. E. W. Price and Mr. Allen McIntosh. The material consisted of 6 specimens in alcohol (bottle M 248-D), the type specimens of *Distomum longum* Leidy, and other specimens mounted on slides and bearing the following labels, U. S. National Museum, Helminthological Collection:

No. 49. *Distomum longum* from *Esox reticulatus*, determined by Albert Hassall; 1 slide. (Plate I, Fig. 3.)

No. 3359. *Azygia lucii* from *Esox lucius*, collected and determined by Professor M. Braun, 7 July 1902, Königsberg, Germany; 1 slide. (Plate I, Fig. 1.)

No. 10500. *Azygia acuminata* from *Amia calva*, type and paratypes; 4 slides. (Plate II, Fig. 9.)

No. 10502. *Azygia bulbosa* from *Amia calva*, type and paratypes; 3 slides. (Plate II, Fig. 11.)

No. 10679. *Azygia loossi* from *Micropterus salmoides*, cotypes; 3 slides. (Plate I, Fig. 6.)

No. 51399. *Azygia sebago* from *Salmo sebago,* 5.2 mm. long, 1 slide, H. B. Ward collection.

No. 51401. *Azygia sebago* from *Perca flavescens,* H. B. Ward collection; 2 slides. (Plate II, Fig. 8.)

No. 51403. *Azygia sebago* from *Amia calva,* 20 mm. long, 1 slide, H. B. Ward collection. (Plate I, Fig. 2.)

No. 51402. *Azygia angusticauda* from *Stizostedion vitreum,* collected by H. W. Manter, 4 April 1926, Rock River, Illinois, 2 slides, H. B. Ward collection. (Plate I, Fig. 7.)

Examination of the specimen of *Azygia lucii,* No. 3359 in the U. S. National Museum, invalidates the criteria used by Ward and Manter to distinguish between *A. longa* and *A. lucii.* In this specimen (Fig. 1) which measures 23 mm. in length, collected and identified by Professor M. Braun, the pharynx is not twice as long as broad; in fact, the organ measures 0.80 mm. long and 0.60 mm. wide. Furthermore, the vitellaria extend far behind the posterior testis; the follicles on the left side about one-half the distance from the testis to the end of the body. In the Ward collection there is a specimen from *Amia calva* (Fig. 2) which measures 20 mm. in length and which resembles the European specimen so closely that I am disposed to regard the two as specifically identical. Leidy, Stafford and Cooper all reported the finding of *A. lucii* and it appears that this species does occur in North America. *Esox lucius,* the type host, is circumpolar in range, and the distribution of its parasites may be expected to parallel that of the host. The dispersal of fishes in the northern hemisphere following the last glacial period has been traced by Walters (1955).

Although the criteria used by Ward and Manter to distinguish *A. longa* from *A. lucii* are inadequate, the two forms are probably distinct. About 100 specimens collected by Mr. Paul Krupa from *Esox niger* in southern New Hampshire during the summer of 1955 are so similar to the six worms in alcohol, now in the U. S. National Museum, which constitute the original material of the species described by Leidy (1851) as *D. longum,* that they must be regarded as identical. A representative example from the Krupa collection is shown (Fig. 5) and a smaller one (Fig. 4). These worms are very slender. The Krupa specimens were dropped in cold Duboscq-Brasil fluid and fixed without narcotization or pressure. Oviferous specimens vary from 4 to 26 mm. in length and 1.1 mm. is the greatest width. The width does not increase very much as the worms grow in length. Comparison of Figure 5 with that of *A. lucii* (Fig. 1) portrays what are believed to be specific differences. Further evidence that *A. longa* is distinct from *A. lucii* is afforded by comparison of the cercariae. *Cercaria mirabilis* Braun, 1891, shown by Szidat (1932) to be the larval stage of *A. lucii,* is very different from the cercaria described by Sillman as the larval stage of *A. longa.* Moreover, in Europe *A. lucii* uses a pulmonate snail, *Lymnaea palustris corvus,* as the first intermediate host, whereas according to Sillman, the asexual stages of *A. longa* occur in the pectinibranchiate snail, *Amnicola limosa.*

Recognition of two distinct species, *A. lucii* and *A. longa,* may resolve certain difficulties and clear up confusion in the literature. The worms from *Esox masquinongy* which Stafford (1904) described as *Megadistomum longum* (Leidy) measured up to five inches in length when extended and probably were not iden-

tical with *A. longa* of Leidy. Stafford reported a specimen 18 mm. long which contained no eggs. Cooper (1915) identified specimens from *E. masquinongy*, which he regarded as identical with those of Stafford, and others from *E. lucius*, as *Azygia lucii*. His specimens from the muskellunge measured 21 to 48 mm. in length and 1.40 to 2.40 mm. in width, whereas those from the pike were 14 to 20 mm. in length and 0.74 to 1.42 mm. in width. Comparison of the small worms from *E. masquinongy* with worms from *E. lucius* led Cooper to regard them as conspecific. But he was unable to account for the variable size at which eggs are produced in different individuals. He reported that a specimen 14 mm. long from *E. lucius* was less mature than another 6 mm. long from the same host species, and that worms from the trout and small-mouthed black bass were all immature although one from *S. namaycush* was 11 mm. long. Discussing the effect of season on sexual maturity, Manter (1926) wrote (p. 67.) ". . . it is certain that what is evidently the same species does not attain sexual maturity at the same time in different hosts in which it occurs. Thus, while average sized forms are producing eggs in such hosts as pike, pickerel, and salmon, specimens fully as large are still sexually immature in such hosts as smelt, trout, small mouthed black bass, and perch." Admittedly, members of a trematode species attain a greater size in a larger host species, and *E. masquinongy* is much larger than *E. lucius*, but present information strongly indicates that *A. longa* is distinct from *A. lucii*, if, indeed, the large American species is actually *A. lucii* of European fishes.

All previous authors have agreed on the identity of *A. angusticauda* (Stafford, 1904) and *A. loossi* Marshall and Gilbert, 1905. A cotype specimen of *A. loossi* (U. S. Nat. Mus., 10,679), shown in Figure 6, is 4.88 mm. long and is obviously young, with only a few eggs in the uterus. A fully mature, gravid specimen (U. S. Nat. Mus., No. 51,402) from the walleye, *Stizostedion vitreum*, collected by Manter in 1926, which measures 10.5 mm. in length, is shown in Figure 7. In both, the acetabulum is near the middle and the gonads are situated in the caudal one-sixth of the body. The distinctness of this species appears to be well established.

The specimens of *Azygia* found in the eel at Woods Hole are clearly distinct from *A. angusticauda* and, as noted, are probably distinct from *A. longa*. Specimens of *A. longa* are slender and much elongate; those from the eel are shorter and more robust. The worms collected by Mr. Krupa from *Esox niger* in New Hampshire and identified as *A. longa* remained well extended when dropped into Duboscq-Brasil killing fluid, whereas those from the eel contracted strongly with the result that the length was only 6 to 8 mm., less than one-half that of *A. longa*. Accordingly, most of the worms from the eel were killed and fixed under pressure, which resulted in longer, wider, and flatter specimens. The size of the suckers increased as a result of the compression but comparison of Figures 10 and 13, which were made from one of the largest and one of the smallest oviferous specimens, with Figures 5 and 4, of comparable specimens of *A. longa*, portrays differences between the two forms which are believed to be specific.

Whereas the worms from the eel differ distinctly from those identified as *A. longa*, they agree almost completely with Goldberger's description of *A. acuminata* and agree almost as well with the descriptions of *A. sebago* as given by Ward and Manter. Certain worms from the eel are very similar to specimens in the Ward collection labelled *A. sebago*. It is probable that Ward had more than one species and that his description of *A. sebago* was based on specimens of both *A. longa* and

A. sebago. The worm from the Ward collection which bears the U. S. Nat. Mus., No. 51,401, shown in Figure 8, is clearly *A. sebago,* and the worm on U. S. Nat. Mus., No. 51,403, from *Amia calva* shown in Figure 2, is so like *A. lucii* (*cf.* Fig. 1), that the two might be regarded as specifically identical. Other specimens of *A. sebago* agree so completely with Goldberger's description of *A. acuminata* (compare Figs. 8 and 9), that I am inclined to regard them as identical. Since Ward probably confused two species in his description of *A. sebago,* the removal of the elongate specimens leaves the description virtually the same as that of *A. acuminata.* Specific determination may be impossible on the basis of adult morphology alone and knowledge of life-cycles and larval stages may be required to finally solve the problem. Why the species occurs only in *Anguilla rostrata* in the Woods Hole area is quite unknown. The larval stages are relatively abundant in the snails of the region, but sexually mature worms have so far been found only in the eel. The chain pickerel, *Esox niger,* is common in these ponds where it has been introduced in stocking operations. Since the worms develop in eels in ponds where pickerel, perch, bass and other fishes are not infected, it appears either that the ecological conditions and food-chain lead to the infection of eels rather than other fishes or else the other fishes do not retain the parasites. In the latter event, a separate species must be involved.

When worms were removed from the stomachs of eels and placed in pond water, the eggs in the terminal coils of the uterus were extruded in a string of mucus. These eggs appeared to be fully embryonated and the miracidium was studied in the egg. Although active, the larvae did not emerge in water and hatching occurred only after the eggs were ingested by the snail host. Empty shells were recovered in the feces of *Amnicola limosa* that had eaten the eggs. Some of these snails were found later to be infected but since they had been collected from locations where previous exposure to infection was liable, it would be difficult if not impossible to distinguish between a natural infection acquired before collection and an experimental one. But the snails laid eggs in the finger bowls and young laboratory-raised specimens were fed eggs of the parasite. These small snails became infected and although emerged cercariae were not obtained before the end of the summer, the developmental stages in these experimental infections were indistinguishable from comparable stages in natural infections. In nature, the eggs of the parasite are passed in mucous material from the intestines of eels and settle on vegetation and on the slimy surfaces of submerged rocks and sticks. The snails rasp these surfaces for the diatoms which form a major constituent of their food and incidentally ingest the eggs. The larvae remain alive for long periods and since the eggs do not hatch until they are eaten, the probability of reaching a suitable host and continuing the life-cycle is much enhanced. The larvae emerge in the intestine of the snail and bore through the wall to reach the haemocoele, where they become sporocysts. Young sporocysts have been found adjacent to the intestinal wall two weeks after eggs of the parasite were added to the finger bowl with the young snails. Older infections with rediae and developing cercariae were found later, which definitely link the experimental and natural infections. However, the rate of development of the parasites and the degree of maturity of the infection are not regarded as significant. It is common knowledge that asexual stages of digenetic trematodes persist but fail to grow or reproduce if the hosts are not fed. Thus, infections overwinter in a quiescent stage in mollusks that are dormant or in

which metabolism is reduced to a low level. In the present instance, although various methods, including those recommended by Moore *et al.* (1953) and by Sandground and Moore (1955) for the rearing of related snails, were employed, it was obvious that the snails, although most of them remained alive, were not properly nourished, did not grow normally, and the tissues had the atrophic appearance typical of inanition.

Cercariae from natural infections were snapped up by guppies and by small bluegill sunfish, *Lepomis macrochirus*, 2 to 4 cm. in length. The young worms were recovered from the stomachs of these sunfish two and three weeks after they were eaten, but there was very little development of the parasites. These small fishes also ate planarians, *Dugesia tigrinum;* so in nature the fishes could contract the infection by eating either the cercariae or infected planarians. The tails of the cercariae cease to beat after about 48 hours and they would then not be attractive to fishes; moreover the larvae die during the next 48 hours. As stated earlier, the young worms live for weeks in the pharyngeal pockets of *D. tigrinum* and this accessory method of employing an additional paratenic or transfer host enhances the likelihood of survival and aids in the completion of the life-cycle. The cercariae are probably not eaten by eels which are at the end of the food-chain that leads to their infection.

Description of Stages in the Life-Cycle

Adult

The worms are only slightly flattened, almost cylindrical, with rounded ends and enormously developed musculature. Because of the ability to extend and retract the entire body or particular regions to an extraordinary degree, measurements of length and width and location of individual organs have limited significance. A specimen may extend to four or five times its length when contracted, and contraction of different regions can make distances between organs so variable that measurements may be very misleading. Ward (1918) wrote (p. 392), "*Azygia* is a powerfully muscular type and is usually much distorted in the process of preservation so that a lot of specimens taken from the same host at the same time present marked external differences in the preserved condition. Such extreme specimens have been the basis for various new genera, *e.g., Megadistomum* of Leidy and Stafford, *Mimodistomum* of Leidy (*sic*) and *Hassallius* of Goldberger. This same factor has lead to the separation of too many as species." Oviferous specimens from the eel, fixed by the shaking method of Looss, are 3 to 9 mm. long and when fixed under pressure measure 4 to 12.5 mm. in length. Because of the variations caused by muscular contractions on the shape of the body and location of organs, dimensions of the suckers and gonads provide the most reliable morphological data, but these organs appear larger in specimens that have been fixed under heavy pressure. Egg sizes vary too much to provide reliable specific criteria. The worms continue to grow after sexual maturity. A large one and a small one are shown in Figures 10 and 13; both were fixed under pressure and are therefore comparable. Measurements in millimeters of the larger one are: length, 12.5; width, 2.2; oral sucker, 0.96; acetabulum, 0.8; pharynx, 0.36 long and 0.32 wide; ovary, 0.54 by 0.23; anterior testis, 0.5 by 0.33; posterior testis, 0.5 by 0.4. Corresponding measurements of the smaller worm are: length, 5.3; width

0.65; oral sucker, 0.41; acetabulum, 0.34; pharynx, 0.19 long and 0.16 wide; ovary, 0.195 by 0.12; anterior testis, 0.195 by 0.143; posterior testis, 0.24 by 0.16. The eggs, alive, averaged 0.06 by 0.034 mm.; under oil immersion and slight pressure, to study the miracidium, they were slightly larger; in fixed and stained worms they were smaller, and averaged 0.055 by 0.030 mm. In such mounted specimens the eggs are usually collapsed and distorted.

Miracidium

The miracidium of *Azygia lucii* was described by von Nordmann (1832), Schauinsland (1883) and Looss (1894) and that of *Azygia acuminata* (possibly a synonym of *A. sebago*) by Manter (1926). The miracidium of the worms identified as *A. sebago*, studied alive in the egg (Fig. 19) and in stained sections of gravid worms, is similar to that of related genera in the family Azygiidae, as reviewed by Manter (1926). Like the others, it lacks cilia and is provided with bristle plates or plaques. It almost fills the egg-shell; the anterior end may be protruded as a conical papilla on which the ducts of the secretory cells open. Radiating from this area, there are five plates or plaques that bear fine bristles arranged in a chevron-like pattern. The anterior ends of the plates are separated by short intervals, which become wider posteriorly. The plaques extend backward about one-third of the length of the larva; the bristles on the anterior portions are larger and longer than those more posteriad. From a naked area at the posterior end of the larva, four bristle-bearing bands extend forward past the middle of the body. The bands are equidistant from each other and both the anterior and posterior ones manifest a spiral tendency, but this aspect may be the result of rotation of the larva within the shell. The appearance of the miracidium is almost identical with that of *Proterometra macrostoma* as reported by Hussey (1945). Hussey described a structure, designated by earlier authors as a "primitive gut", with four nuclei arranged in a linear series. In *A. sebago*, the corresponding structure, which is glandular and probably serves in penetration, consists of four cells which lie side by side rather than in linear series. These cells are disposed as reported by Manter (1926) for the miracidia of *Otodistomum cestoides, Otodistomum veliporum* and *Azygia acuminata*. Manter reviewed previous accounts and presented a strong argument that the organ is not a primitive gut, but a group of unicellular glands. Immediately posterior to the glandular organ there is a bilobed "brain" and the region behind it contains several large, germinal cells. On either side, near the middle of the body, there is a single flame cell from which an excretory tubule leads caudad, but the ducts were not traced to the pores.

Asexual generations

The youngest sporocyst was recovered from a loose network of connective tissue on the somatic side of the intestinal wall of a laboratory-raised snail that had been exposed 12 days previously. It was oval, 0.094 by 0.062 mm., with no lumen; it contained germinal cells but no germ-balls (embryos). Other larger sporocysts were found in older infections; one, 0.126 by 0.08 mm., contained germinal cells and 6 small germ-balls; another, 0.189 by 0.12 mm., contained germinal cells and 9 germ-balls of varying sizes. In a snail killed one month after exposure, the mother sporocyst could not be recognized but there were 26 rediae scattered about

in the haemocoele. The smallest was 0.25 by 0.18 mm., and in addition to germinal cells it had four small spherical to oval germ-balls, 0.02 to 0.04 mm. in diameter. A redia with larger germ-balls but no recognizable cercariae measured 0.57 mm. long and 0.18 mm. wide; the pharynx was 0.08 mm. in diameter and there was a sac-like gut, 0.11 mm. long and 0.032 mm. wide. The largest redia was 1.3 by 0.3 mm. and in addition to smaller embryos, it contained two cercariae, one of which was more than half-grown and had small furci. Whether or not there is a second generation of rediae was not determined.

The cercaria-producing generation of species in the genus *Azygia* was recognized by Szidat (1932) as redial, although the pharynx undergoes reduction to a mere vestige and the intestine completely disintegrates. As noted by Szidat in *A. lucii*, the pharynx, which he termed "rudimentary", serves for the ingestion of bits of the digestive gland of the host and persists as a birth-pore through which the cercariae emerge. The small rediae are vermiform and very active; the pharyngeal end may be inrolled and then everted, while the opposite end may be protruded as a pointed, tail-like structure. Older rediae may extend to a length of 3 mm. and on contraction of the circular muscles, present an annulate appearance. On contraction of the longitudinal muscles they become oval and about 1 mm. in width. The one shown in Figure 20 is bent and as mounted measures 1.12 by 0.325 mm.; in it the pharynx is still distinct. The older, larger, rediae have little mobility but pulsations of one and sometimes two can occasionally be seen through the shell of an infected snail. The number of cercariae in a redia is small; often there is only one and rarely are there more than three recognizable cercariae; other individuals are still in the germ-ball stage, together with a few germinal cells attached to the body wall, chiefly at the posterior end of the redia. Apparently the development of one cercaria restrains the development of others. An infected snail may liberate one or two cercariae each day for a few days and then none for a week or more. The large size of the cercariae is correlated with the slow development and the small number produced.

Cercaria

Developing cercariae are typical furcocercous larvae. As the embryo reaches a length of approximately 0.25 mm., a constriction appears and gradually separates the posterior one-fourth to one-third of the larva as an oval, tail-rudiment. At about this stage, the oral sucker is faintly outlined. When the larva has reached a length of 0.4 to 0.5 mm., the suckers are distinct, the acetabulum is in the posterior half of the body, the tail is about three-eighths of the total length, and the furcal buds are beginning to appear. As development proceeds, the tail increases in length more rapidly than the body; its basal portion, about one-sixth of its length, begins to enlarge and by the time the gonads are recognizable, the anterior end of the tail forms a cup-like ring (Fig. 20), at the base of which the constricted caudal end of the distome is continuous with the tissues of the tail. The cercariae complete their growth in the rediae and emerge into the haemocoele of the snail. While studying the excretory pattern of a redia which was under some pressure, an immature cercaria emerged, tail first, through the old pharyngeal opening. The cercariae mature in the haemal sinuses of the snail, especially the branchial sinus, and emerge through the respiratory opening. During growth, the basal portion of the tail is much en-

larged by the accumulation of spongy, fibro-elastic, alveolar tissue which, when the cercaria emerges from the snail, absorbs water and expands rapidly. As a result, this portion of the tail extends forward, encapsulating the body of the cercaria. If infected snails are crushed and immature larvae are liberated into water, the base of the tail is unable to completely engulf the body of the larva (Fig. 18).

Mature, normally emerged cercariae measure 1.8 to 2.3 mm. in length. The expanded, basal portion of the tail is flattened, 0.5 to 0.75 mm. in width, and slightly more in length. The stem of the tail, that portion from the spongy, rigid, basal part to the furci, is 1.0 to 1.5 mm. in length and 0.26 to 0.46 mm. in width. It tapers slightly from the basal to the distal end. It is distinctly flattened and set at right angles to the dorso ventrally flattened body of the larva, so that when looking at the flat aspect of the tail, the body appears in lateral view (Fig. 17). This stem portion of the tail consists of two bands of longitudinal muscles, one on each of the flat surfaces. These muscles are attached at one end to the rigid, spongy portion of the tail and at the other end to the bases of the furci. The furci are flattened, 0.55 to 0.90 mm. in length and 0.20 to 0.28 mm. in width. Normally they are held almost at right angles to the tail stem, whose muscle bands contract alternately, so that the flapping of the tail from side to side produces a sculling effect that pulls the larva through the water. After the beat of the tail is unable to lift the larva from the bottom, it continues for a day or two and this flapping motion makes the larva an attractive lure for small fishes and perhaps other predators. The basal end of the tail becomes sticky and may lightly attach the larva to the substratum. How the larvae reach the pharyngeal cavity of the planarians is not clear. The body is firmly enclosed in the chamber at the anterior end of the tail and could be liberated only by dissolution of the tail. According to Hyman (1951, p. 107), "The triclads do not swallow their food whole but suck it in by peristaltic action of the protruded pharynx." and (p. 199), "The Turbellaria are as a class carnivorous. . . . Favorite items of food of the smaller species are rotifers, copepods, cladocerans, nematodes, annelid worms, etc.,". Perhaps the planarian seizes the larva, and as the tail is sucked in and digested, the young worm is liberated and attaches to the external surface of the pharynx, whence it is carried into the cavity when the pharynx is retracted.

The tail bears many papillae, scattered somewhat irregularly over the surface except for the distal three-fourths of the furci. Each is about 0.05 mm. in diameter, 0.025 mm. tall, and is surmounted by a recurved hook, 0.012 to 0.015 mm. in length. The tail also has many opaque patches, which on higher magnification are seen to consist of minute spherules. The excretory system of the larval body is continuous with that of the tail and the constricted caudal end of the body contains the common excretory canal which traverses the stem of the tail, bifurcates at the bases of the furci, and the resulting tubules open at the tips of the furci. The pattern of flame cells in the tail was not resolved.

The morphology of the young worm, released from the chamber in the tail, is typically azygiid (Figs. 15, 16). The cuticula is unarmed but the preacetabular region bears many papillae and a bristle has been observed at the tip of certain of them. There are at least a dozen papillae, 0.018 to 0.020 mm. in diameter, around the anterior end of the worm. Living specimens vary from 0.7 to 1.3 mm. in length and 0.16 to 0.28 mm. in width. The acetabulum varies from 0.10 to 0.13 mm., and the oral sucker, 0.11 to 0.14 mm., in diameter. The pharynx measures 0.05

to 0.07 mm. in length and usually slightly less in width. The digestive ceca are filled with yellow material, derived from the digestive gland of the snail. The excretory system is complex but has been worked out completely. The pore is terminal and a common duct leads forward almost to the level of the testes. The posterior one-half of this duct may expand to form a bladder-like enlargement, or if the pore is blocked and fluid accumulates, the enlargement may extend farther forward. Behind the testes the common duct divides, forming two ducts which pass forward, median to the digestive ceca. As the ceca turn mediad to join the pharynx, the excretory ducts pass below them and continue on either side of the oral sucker almost to the anterior end of the body. There is, however, no connection between the ducts of the two sides. Anterolateral to the oral sucker, the duct of each side doubles backward and continues posteriad, giving off eleven branches. Each branch divides three times, forming two primary, four secondary and eight tertiary branches. Each tertiary branch receives the capillaries from four flame cells. The flame cell formula accordingly is 2 (11 × 32) or 704 flame cells in the body. This observation is in agreement with that of Looss (1894) who described the same pattern in *Azygia tereticolle* (= *A. lucii*). He regarded the ascending portions of the excretory system as parts of the excretory vesicle and the descending limb with its branches as the collecting ducts. He suggested the possibility of variation in the number of branches and of anastomoses between collecting ducts; however, I have found a constant number of branches and the apparent anastomoses can be resolved as places where one duct crosses another. Counting backward from the anterior end of the body, the first side branch is located at the level of the oral sucker; the second is at the level of the bifurcation of the digestive tract, *i.e.*, the posterior end of the pharynx; the third branch is anterior to the acetabulum; the fourth is at the middle of the acetabulum; the fifth is at the level of the posterior end of the acetabulum; the sixth and seventh are close together a short distance behind the acetabulum; the eighth, ninth and tenth are almost equally spaced; while the eleventh and last, which is the terminal group of the recurrent limb, is distributed to the extreme posterior end of the body around the excretory bladder. The reproductive organs are represented by groups of deeply staining cells, shown in Figures 15, 16 and 17.

Summary

A chronological account of the genus *Azygia* discloses discordant observations and divergent opinions. Dawes (1946) recognized only a single species, *A. lucii*, in Europe. In it he included *A. robusta* Odhner, 1911, which reaches a length of 47 mm. and *Ptychogonimus volgensis* von Linstow, 1907, which measures 5 to 6 mm. in length and had been transferred to *Azygia* as a valid species by Odhner (1911). In America several species have been described, but there is no agreement on the number that are distinct and valid. In fact, there is no adequate information on the extent of variation that occurs in a natural species, and consequently on the features that can be relied on to distinguished between species. This situation is not peculiar to *Azygia*, but obtains in many genera. It is the natural result of development by members of a parasitic species in different hosts, invertebrate and vertebrate, often of different taxonomic groups, which differ in their nutritional and other physiological conditions, and accordingly influence the development and

morphological features of the parasite. Until the life-cycle is known and the variation that normally occurs in each possible host is measured, the precise limits of specificity will remain uncertain. Comparison of specimens and descriptions indicates that *A. lucii* may be endemic in North America, that possibly it is distinct from *A. longa* (Leidy), that *A. angusticauda* (Stafford) is a valid species, and that the species described by Goldberger (1911) may be identical with *A. sebago* Ward. Information concerning the life-history of species in the genus *Azygia* is meager. Szidat (1932) showed that *Cercaria mirabilis* Braun is the larva of *A. lucii*. He described a second larva, *Cercaria splendens*, presumably another species of *Azygia*, but the adult stage remains unknown. Sillman (1953a) reported the life-cycle of a species that he identified as *A. longa* and the present paper presents data on the morphology and life-history of a species believed to be *A. sebago*. Stages in the cycle are described and figured.

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TWINNING AND REPRODUCTION OF TWINS IN PELMATOHYDRA OLIGACTIS

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This paper is written to extend the observations of the writer (Turner, 1950; 1952) on the reproductive potential in clones of *Pelmatohydra oligactis* and the regulation of spontaneous structural anomalies in the same species. A review of relevant literature and certain conclusions drawn from the literature are contained in the two articles and they will not be repeated here except for a few citations bearing directly upon the subjects of longitudinal fission and regulation.

Hyman (1928) states (p. 73) "that fission in hydra is not a normal method of asexual reproduction but a mode of regulation of previously existing abnormalities." The writer (Turner, 1952) observed (p. 107) that "the bifurcated condition of the apex which initiates longitudinal fission originates most commonly, and possibly exclusively, in buds which are in the latter stages of development and are still attached to the parent." Chang, Hsieh and Liu (1952) found that fission occurred in buds in a ratio of about one in a thousand. The writer has found since 1952 in large mass cultures and also in isolated strains that twinning in buds is fairly common and that true twinning originates only in buds. Conditions resembling twinning have been discovered in mass cultures but when these specimens are followed through day by day some of them have proved to be cases of actual twinning but others have proved to be specimens undergoing regulation. The complexes are often resolved by fusion of the members, absorption of one member by the other or one member forms a new foot and separate from the other.

The term "twinning" is used here advisedly and has most of the features common to monozygotic twinning in higher forms. In both hydra and higher forms an embryonic mass, produced asexually as a bud in hydra, divides and two individuals proceed separately to develop into whole equal animals. The separation of the two individuals may be incomplete in higher forms and produce monstrosities in various degrees. In hydra, individuals will become separated eventually because they have retained to a remarkable degree the capacity for regulation during which multiple complexes are reduced to simple individuals with the characteristics of the parent animal. In hydra the initiation of twinning may occur at any stage of bud development but it is not completed by the time of detachment from the parent. Another process comparable to twinning in its end result is double budding. In this process two buds in precisely the same stage of development arise at the same time in the budding zone of the parent as separate masses, usually on opposite sides of the body stalk, and after developing at the same rate they become detached at the same time. At no stage are they connected and they differ from the ordinary bud in that ordinary buds arise in a sequence, differ from each other in stages of development and become detached from the parent at different times.

Materials and Methods

Specimens were separated from a large mass culture and were placed individually in bottles of about 100 cc. capacity. They were fed with Entomostraca daily and the water in which each lived was replaced daily with water from the large tank in which the mass culture was maintained. Daily records were kept of each specimen concerning its behavior, its reproduction and its periods of physiological depression.

In order to secure an accurate determination of the extent of twinning in the members of the mass culture, single non-twinning specimens were separated from the mass culture and a record was kept of all of the young produced by budding. The record of each was followed for 20 to 30 days and the proportion of twinning to non-twinning buds was determined from the totals. The process was repeated three times at two-month intervals.

When twinning buds were discovered they were isolated after they had become detached from the parents and daily records were kept of buds produced by the single undivided portions and by the bifurcated portions of the twin. The total number of buds produced by the complex was secured in this way and a comparison could be made between twinning individuals and single individuals of the rate of bud production. The proportion of single to twinning buds was obtained and could be compared to the production of single and twinning buds in control specimens which had no previous history of twinning. The possibility that certain strains had a tendency to produce a high proportion of twinning buds could be examined from the results.

Proportion of Twinning to Non-twinning Individuals

Any estimate of the proportion of twinning to non-twinning individuals based upon bifurcated specimens taken from a large mass culture would be invalid because a part of the individuals in the bifurcated state would not be cases of genuine twinning. Some of them would be mature individuals in which a bifurcation had arisen at the apex during or immediately after a period of depression. By the selection of healthy single individuals and the recording of each bud which arose in these individuals, it was possible to know whether any suspected case of twinning was real and, if it was real, to know also the place of its origin and its subsequent history. Twenty-five single individuals selected from the large mass culture were given the most favorable conditions for reproduction and within a period of 20 to 30 days they produced a total of 705 buds of which 14 were twins. The twinning buds represented 1.98 +% of the total. Two months later 11 single individuals, selected from the same mass culture and maintained in the same way, produced 274 buds of which 4 (or 1.45 +%) were twins. The operation was repeated two months later and 12 specimens produced 299 buds of which 6 (or 2.01%) were twins. In the three samples, 48 isolated individuals produced 1278 buds of which 24 or 1.87 +% were twin buds.

Twenty of the twin buds were isolated when they were detached from the parent and a record was kept of the production of buds by each specimen for 13 to 22 days. A total of 523 buds was produced of which 19 (3.63 +%) were twin buds. During this time ten of the twinning specimens produced only single buds but one of them produced three twinning buds (Fig. 3) and several produced two twinning buds

(Figs. 1 and 2). Since the reproducing individuals derived from twinning buds produced nearly twice as many twinning buds as individuals taken at random from the mass culture, it may be inferred that an inherited tendency for bud twinning existed in the strains showing unusual bud twinning.

Origin and Structure of Twins

Twinning has been observed in buds at various stages of development. In the earliest cases the bud develops a bilobed condition almost from the moment of its origin as a bud (Fig. 17). Each lobe then elongates and differentiates (Figs. 4 and 5) and by the time of detachment from the parent the two members of the twin are separate except at the base (Fig. 6). If the specimen indicated in Figure 6 were to be found in a mass culture without its previous history being known, it might be interpreted as a case of apico-basal fission but it is actually one in which no fission is involved up to the time of its detachment. Rather, two separate portions of the original single bud have undergone parallel development. After detachment the twinning individual undergoes fission and the two members are separated. It may be stated that twinning gives rise to an anomalous state which is resolved by regulation (fission).

A bud may develop as a single unit for some time and then give rise to dual masses at the apex (Fig. 7). The basal single portion elongates somewhat and at the same time the members of the divided portion will elongate and differentiate. After detachment from the parent (Fig. 8) fission of the remaining common stalk and the base occurs. Regulation (fission) is involved to a greater extent than it is in cases of very early bud-twinning.

Twinning occurs at a late stage of bud development in some cases (Fig. 9) and when the bud is detached from the parent only the apical portion of the bud is bifurcated. The regulation process (fission) occupies a longer part of the entire period during which two complete individuals are formed from a single bud.

A bud occasionally gives rise to three instead of two units. In the case illustrated in Figure 11 three hypostomes, each with a circle of tentacles, arose in a late stage of bud development. Before the bud was detached the hypostome of one unit was absorbed by another but the tentacles of the absorbed unit remained intact. When the bud was detached it resembled the one shown in Figure 10 except that one of the terminal units possessed supernumerary tentacles. In this case three processes of regulation would be involved before two normal single individuals were formed from the complex bud. Absorption of the hypostome occurs before bud detachment. Apico-basal fission would separate the two members after detachment of the complex from the parent, and still later fusion of tentacles would occur in the individual having supernumerary tentacles until the normal number of tentacles was produced.

Secondary twinning, *i.e.*, twinning in one or both members of a specimen which is itself a twin, has been observed a number of times. In Figure 12 a single speciment is illustrated together with a twinning bud. The primary twinning occurred when the bud was half developed and the two members of the twin bud proceeded to elongate and differentiate. At a late stage in differentiation one of the members divided at the apex. When the bud was detached it consisted of a common stalk and foot and a stalk divided in its terminal half. Also one member of the di-

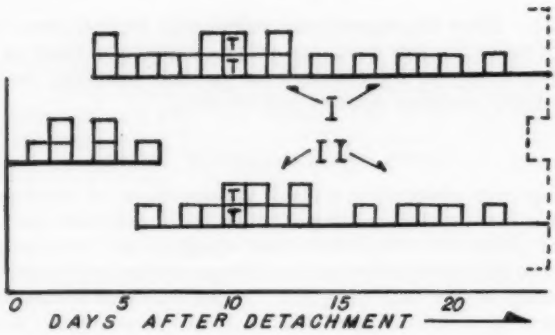


FIGURE 1

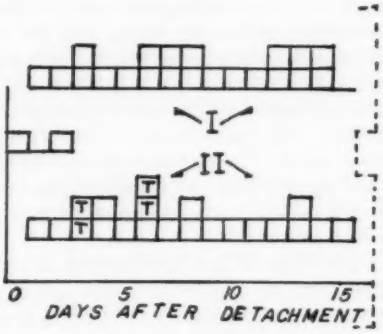


FIGURE 2

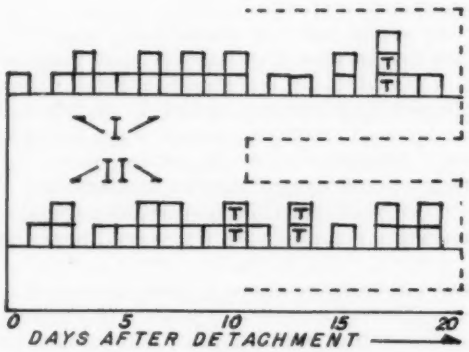


FIGURE 3

FIGURE 1. Reproduction by budding in twinning specimen shown in Figure 10. Period covers time from detachment from parent to time of separation of twins by apico-basal fission. Fission was completed in 24 days. Squares indicate new buds per day. Reproductive record of single portion shown at left, of divided portions, in I and II. T indicates twinning in a bud.

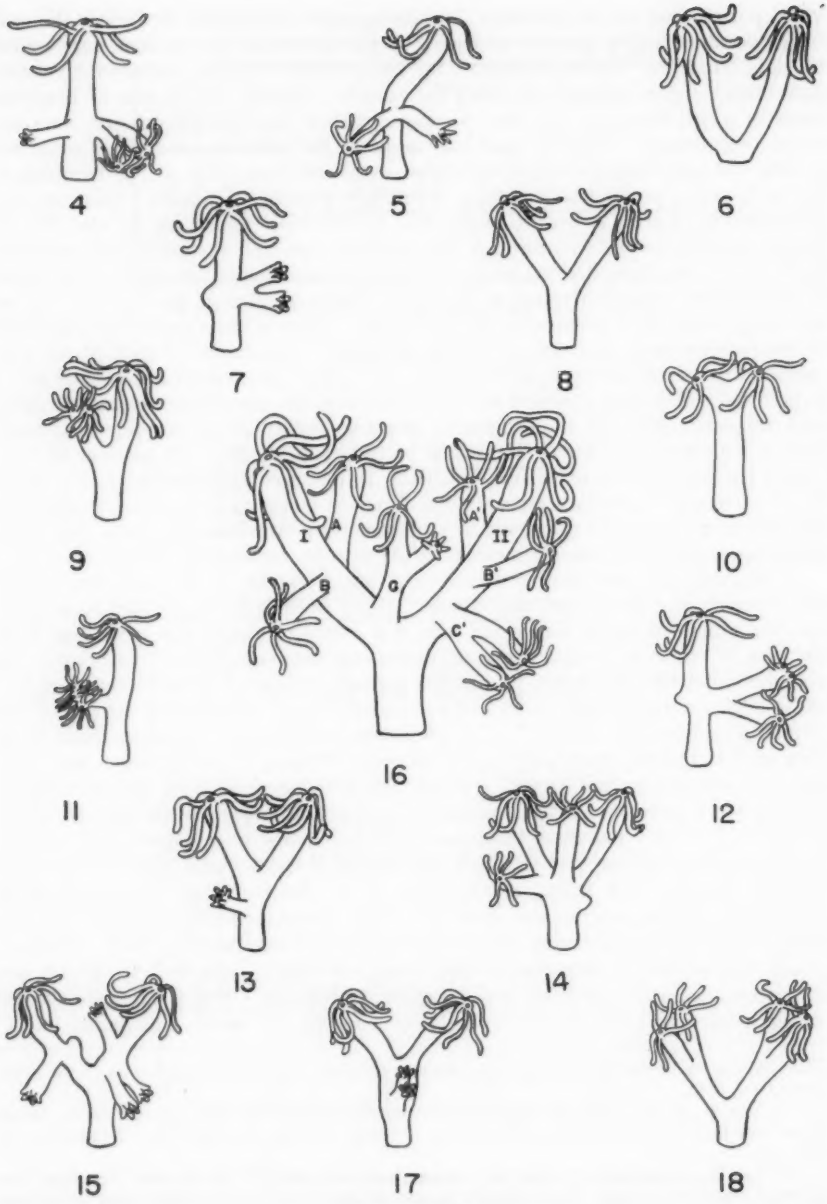
vided portion had two hypostomes. In the member which was divided at the end, fusion occurred and within several days a single hypostome was present, surrounded by eight tentacles. Fission separated the two primary members eighteen days after detachment of the twinned bud from the parent. Figures 15, 16 and 17 illustrate cases in which twinning buds had become detached from the parent and were actively reproducing. Fission which will separate the primary twins is in progress. A new bud appearing on one of the stalks of the specimen (Fig. 17) is twinning in an early stage producing a case of secondary twinning. Similar situations are illustrated in Figures 15 and 16 except that in both cases fission has divided the detached primary twin down through the budding zone and the secondary twinning buds are arising from divided instead of the common undivided parts of the stalk. A remarkable case is illustrated in Figure 18. The complex of four members arose as a single bud which twinned at an early stage. After a short period of elongation of the two members each member twinned again. Elongation of each of the four members occurred and each differentiated with a full complement of tentacles and a hypostome. After the complex was detached from the parent the primary division was completed by fission and some days later a secondary fission separated the members of the secondary twins. No fusion of any kind occurred. It appears in comparing the cases shown in Figures 9, 10 and 18 that secondary twinning is likely to be effective and to produce separate individuals if it occurs early and the members are able to grow and to differentiate a full complement of normal parts, but if the twinning occurs very late one member is likely to be absorbed by the other.

Complexes are encountered occasionally which involve at the same time multiple twinning and other unusual features. They present a complicated appearance and the units can be accounted for only if a complete record has been kept from the time of origin of the individual producing the complex. A case in point is the complex illustrated in Figure 16, and the history of this case is as follows. A bud arose from a normal single hydra and proceeded to twin when half developed. The twinning bud became detached from the parent and began to produce buds of its own in the common stalk almost immediately. Fission of the twinned individual was carried basally and passed through the budding zone. At the moment illustrated in Figure 16, I and II represent the divided portions of the individual described. Portion I has three buds, A, B and C. Bud C is going through the unusual process of giving rise to a new bud before it has become detached from the parent. Portion II is giving rise to buds A′, B′ and C′. Bud C′ twinned at an early stage and has not become detached. The subsequent history of the complex involved further reproduction and a resolution into single normal units. Portions I and II gave rise to new buds while they were being separated by fission and thereafter appeared as normal single budding individuals. Bud C became detached within a few hours and became a single reproducing individual. Twinning bud C′

The notch in the broken line at the right indicates the time of separation by fission of the members of the twin.

FIGURE 2. Reproduction by budding in twinning specimen divided for half of its length when detached from parent as in Figure 8. Fission complete on 16th day. Symbols as in Figure 1.

FIGURE 3. Reproductive record in twinning specimen divided for ⅞ of its length at time of detachment from parent. Reproductive period extends from time of detachment of twinned bud from parent (0 days) to completion of fission (10 days) and in addition, 10 days after completion of fission. Symbols as in Figure 1.



FIGURES 4–18.

became detached in several hours and nine days later fission had separated the members of the twin. In the meantime twinning bud C′ was budding off new individuals. A total of eighteen days were required from the time of the appearance of the original bud for the formation of the complex and for its resolution into single individuals.

Reproduction in Twins

Reproduction in twins was studied for the purpose of determining whether the process of twinning and subsequent regulation interfered with, or in any way affected, the process of reproduction and also to compare the over-all production of new individuals in twins with that of single individuals. Three cases were selected for illustration on the basis of the degree of apico-basal division in the twins at the time of the detachment from the parent.

In case 1 (Fig. 1) the twinning bud was detached from the parent when the apical part was divided for approximately one-third of the entire length of the bud. The budding zone in the common stalk was not divided at the time of detachment and three individual buds were produced within the first four days on the common stalk. Buds produced by the common stalk are shown by blocks in the middle line of the three lines of blocks. On the fourth day after detachment, fission

Figure 4. Single parent with bud which twinned at an early stage and developed as two individuals separated except for basal ⅛ at time of detachment.

Figure 5. Single parent with bud which twinned at an early stage and was almost completely divided at time of detachment from parent.

Figure 6. Bud shown in Figure 4 after detachment from parent.

Figure 7. Young bud which twinned when half developed.

Figure 8. Bud shown in Figure 7 after detachment from parent.

Figure 9. Bud which twinned at a late stage of development. Supernumerary tentacles and fusing pairs of tentacles in parent indicate that parent is an incompletely regulated specimen in which two apical units have fused. Bud was divided for ⅛ of its length when detached.

Figure 10. Bud shown in Figure 9 after detachment from parent. Apico-basal fission was completed in 24 days. See Figure 1.

Figure 11. Multiple division of the apical end of a bud. After detachment one of the hypostomes fused with the nearest hypostome. The two remaining units were separated by apico-basal fission.

Figure 12. Twinning occurred at an intermediate stage of bud development and secondary twinning occurred late in the development of one of the units. The secondary twinning was reduced by absorption of one member by the other.

Figure 13. Budding in the undivided portion of a specimen which twinned as a bud and is undergoing apico-basal fission. See Figure 2.

Figure 14. Same specimen as in Figure 13. Apico-basal fission has proceeded down to the budding zone.

Figure 15. Same specimen as in Figure 13. Apico-basal fission has proceeded through the budding zone down to the stalk. One of the new buds is a twin.

Figure 16. Specimen 8 days after it was detached from parent as a twinned bud divided for about ½ of its length. Apico-basal fission has proceeded downward through the budding zone. Member I has three buds, A, B and C. C, not yet detached, is producing a bud. Member II has produced three buds, A′, B′ and C′. C′ is a twinning bud. Specimen produced 43 buds before fission was completed in 21 days.

Figure 17. Specimen which arose as a twinned bud and is now producing a twinning bud. Divided members of the new bud have unusual arrangement along apico-basal axis of parent.

Figure 18. Complete double twin. Specimen twinned in an early bud stage and each member twinned at a later stage before the four-member complex was detached from the parent.

had carried down to the budding zone and two new buds appeared on the common stalk and two upon one of the divided portions above the point of function with the other member. On the fifth day after detachment another bud appeared on the same member. On the sixth day one new bud appeared upon the common stalk and one each on the divided members. Thereafter no new buds appeared upon the common stalk and it is apparent that fission had carried down through the budding zone and that the time for its passage through the budding zone was three to four days. The time of the complete separation of the members of the twin was 23 days after the twinned bud had become detached from the parent. The time of separation is indicated by the notch in the broken line at the right of the figure. During the process of fission, during which the members were separated from each other, reproduction continued in each member at approximately the same rate as it would have occurred in a single individual.

Reproduction in a bud which had twinned somewhat earlier than the one shown in Figure 1 is represented in Figure 2. When the bud was detached from the parent it was divided apically for about one-half of its length, presenting the appearance of the specimen shown in Figure 8. The division point was within the budding zone and each member began to form new buds the day after the twin was detached from the parent. Fission carried down through the budding zone within three days during which time the common stalk produced two buds. The members of the twin were separated from each other 16 days after the twinned bud was detached from the parent and each produced buds continually during the regulatory process of fission.

A twin-bud specimen which was divided for about seven-eighths of its length when detached from the parent reproduced as shown in Figure 3. The specimen (Fig. 6) had twinned at an early stage and separate and complete budding zones were represented in each member. Each member began to give off new buds as soon as the specimen was detached from the parent and continued to do so for the ten days required for fission to separate the members. The reproductive history of each member for ten days after separation is shown at the right in the diagram. It will be noted that the rate of bud production in each member was about equal and that the rate is the same whether the members were attached to each other or separated.

It is apparent from the results that the process of twinning, and of fission which separates the members of a twinned individual after its detachment from the parent, do not affect the process of reproduction by budding. It may be added that there is no interference with the formation and maturing of spermaries. Buds, whether single or twinning, become detached from the parent within 48 hours after their first appearance if the parent is not in a state of depression, and spermaries are not formed until later. However, spermaries have been observed in both members of twinned specimens during the process of fission which later separates the members of the twin.

Duration of Fission

Fission, as the term is used here, refers to the process moving in the apico-basal axis by which the members of a twinned individual are separated. It occurs after the bud has been detached from the parent. The rate at which fission proceeds is

quite variable and the duration of the process depends upon the degree of initial separation of the members at the time of detachment from the parent and upon the rate at which it proceeds. Four individuals divided at the apical end for a distance of one-fifth of the total length required, respectively, 17 days, 27 days, 38 days and 51 days for complete separation. The process moved through the apical third rapidly, and proceeded through the budding zone in three to four days. Fission moved at a slower rate through the body stalk at the basal end and lagged greatly in the region of the foot. In an extreme case 20 days were required for separation of the foot after fission had carried down to that point. Two specimens, each separated for one-half of the total length at detachment, required 23 days for the completion of fission. Three specimens divided almost to the base at the time of detachment required, respectively, 8, 9 and 11 days for complete separation.

Summary

1. In the specimens observed in pedigreed cultures, genuine twins arose only in buds.

2. Fission, regarded as a separate regulatory process in which twinning complexes are resolved into single individuals, occurs after the twinning buds have become detached from the parent.

3. In pedigreed cultures specimens arising as single buds produced 1278 buds of which 24 were twin buds. Specimens arising as twinning buds produced 523 buds of which 19 were twin buds.

4. Twinning may occur in a bud at any stage of development. An early twinning bud is deeply divided at the time of detachment from the parent and a late twinning bud is divided only at the apex.

5. Multiple twinning occurs occasionally in which one or both members of a twin bud undergo secondary twinning before detachment of the complex from the parent.

6. Bud production by a specimen arising as a twin bud is equal to that of a single individual as long as the budding zone is undivided. Bud production is doubled as the budding zone is divided by fission.

7. Completion of fission of a twin bud requires usually from 8 to 27 days but may take as long as 51 days in a depressed specimen. Fission proceeds rapidly at the apical end, passes through the budding zone in three or four days and is retarded most at the basal end of the body stalk and the foot.

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PROPERTIES OF THE CONNECTIVE TISSUE SHEATH OF THE COCKROACH ABDOMINAL NERVE CORD [1, 2]

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Hoyle (1952, 1953) has drawn attention to the continuous sheath which surrounds nerve fibers and ganglia of *Locusta* and other insects. He has described the structure of this sheath and demonstrated that its effectiveness as a diffusion barrier enables the nerves of *Locusta* to function normally despite wide variations in the ionic composition of the surrounding fluid. His valuable work indicates basic similarity in the membrane properties of insect nerve and nerve of vertebrates and of invertebrates other than insects. In the present study of the ventral nerve cord of the roach, Hoyle's conclusions are confirmed. Normally sheathed and desheathed cords were compared with respect to interference with nervous function by variation in total salt concentration, sodium deficiency, excess potassium ions and acetylcholine. Certain structural details were studied histologically.

Materials and Techniques

Adult male specimens of *Periplaneta americana* have minimal fatty deposits about the cord and were therefore used. For observations of the effects of ions on axonic conduction in the ventral cord, the head was crushed, and the cockroach was pinned, ventral side up, on a cork platform, with the legs taped down. Test solutions were applied and conduction examined in a segment of nerve cord comprising the fourth abdominal ganglion and the connectives between the fourth and fifth ganglia. Cuticle was removed over this region and a thin paraffin sheet was placed beneath the test segment (Fig. 1a). Drainage arranged from below the paraffin minimized mixture of hemolymph with the test solutions, which were perfused over the segment lying on the paraffin. In some experiments, it was possible to avoid cutting any large nerves or tracheal branches by locating the paraffin entirely under the connectives. Silver-silver chloride hook electrodes (Roeder, 1946) were placed below the cord and moved over the test area so that localized axonic block could be detected by changes in form of the compound action potential in the giant fibers (Fig. 1b).

Action potentials conducted into or through the test area could be elicited either directly via stimuli from a pair of silver electrodes (Fig. 1a, S_1) inserted under the nerve cord through a small cuticular opening near the first abdominal ganglion, or transynaptically via stimuli from a similar pair (S_2) inserted into the base of a cercus. An uninterrupted sequence of square pulses (0.5 per second; 0.2 msec.

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[2] The authors wish to acknowledge the able assistance of Miss Janice Green and Mr. Edward L. Raymond in the histological work.

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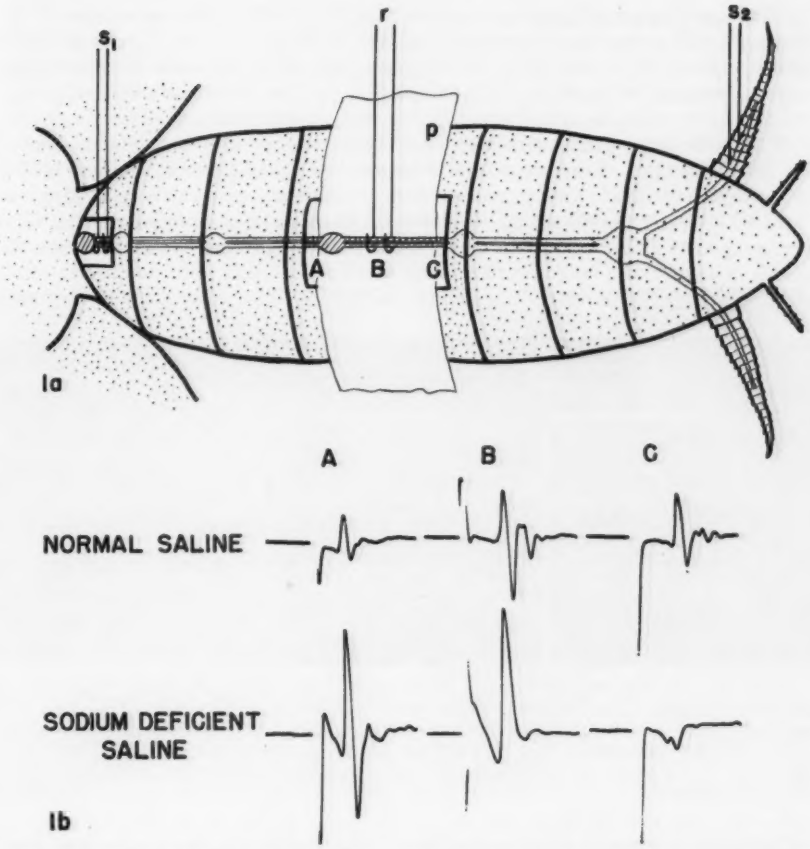

FIGURE 1. (a) S_1, stimulating electrode pair on cord near first abdominal ganglion; S_2, stimulating electrode pair on cercal nerve; p, paraffin sheet; r, recording electrode pair; A, B, C, sites in test area at which action potentials were recorded. (b) Records of action potentials at sites A, B, and C; stimulation at S_1 in a preparation desheathed at B. In sodium-deficient saline, the spikes are larger, due to decreased shunting by electrolyte. Localized block in the desheathed region between electrodes is indicated by the monophasic spike at B.

duration) was applied through S_1 except for brief periods when ascending conduction was checked through stimulation at S_2. In normal or potassium-free saline, this preparation responded uniformly well for many hours. "Normal" saline refers to Hoyle's (1953) basic mixture for *Locusta*, which proved most satisfactory in our experiments.[4] High potassium and potassium-free salines were made up as detailed by Hoyle.

[4] KCl 10 mM./L. $MgCl_2$ 2 mM./L.
NaCl 130 mM./L. NaH_2PO_4 6 mM./L.
$CaCl_2$ 2 mM./L. $NaHCO_3$ 4 mM./L.

The last abdominal ganglion, exposed as described by Roeder, Kennedy and Samson (1947), was the test object in acetylcholine studies. Electrical stimuli were applied to the cercal nerve at low frequency (0.5/sec.) and the postsynaptic response was led off near the fifth ganglion. The last ganglion was continuously perfused with saline, to which acetylcholine was added for tests.

The recording system consisted of a Grass P-4 preamplifier and a Dumont 304-A oscilloscope. Responses were photographed with a Dumont oscillograph record camera, type 297. Square pulses were delivered from a Grass S-2 stimulator.

Methods were developed for desheathing ganglia and connectives in the above preparations. These operations were most conveniently performed under a dissecting binocular at a magnification of 80 ×, using two pairs of fine-ground watchmaker's forceps. Lowering the saline level briefly caused a barely perceptible

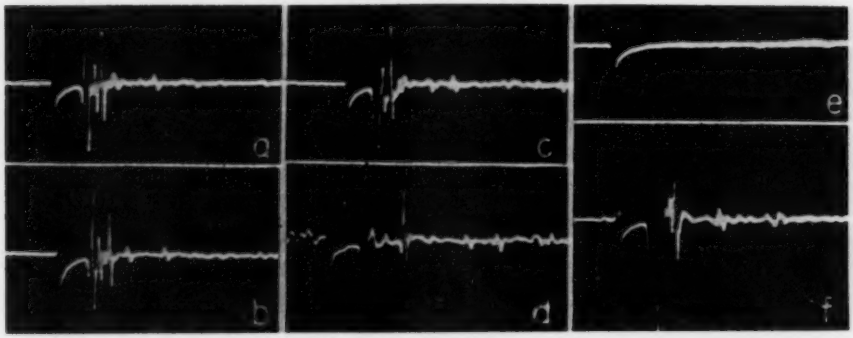

FIGURE 2. Cercal nerve stimulated. Postsynaptic responses recorded from abdominal cord near fifth ganglion. (a) In normal saline; last abdominal ganglion normally sheathed. (b) After 10 minutes in acetylcholine 10^{-3} *M;* normally sheathed. (c) After one minute in acetylcholine 10^{-3} *M;* desheathed. (d) After three minutes in acetylcholine 10^{-2} *M;* desheathed. (e) After four minutes in acetylcholine 10^{-2} *M;* desheathed. (f) After washing in normal saline; desheathed.

crinkling of the sheath about the ganglion. The sheath was lifted, torn, and gently pulled away from the entire dorsal surface of the ganglion. The saline level was rapidly adjusted to prevent drying of the desheathed ganglion. This procedure in no way altered the character of the postsynaptic response (see Fig. 2). Desheathing the connectives was more difficult since conduction failed if even a brief drying occurred, and visualization of the sheath under saline took some practice. Stretching the desheathed connective had to be avoided. The sheath, once torn, could be rolled back along the connective, which it enveloped in stocking-like fashion. The sheath is quite elastic and constriction of the cord by the rolled-up sheath had to be avoided by proper tearing. The stripping, successfully accomplished, did not alter the nerve action potential. In normal saline, desheathed preparations responded without change for hours.

The posterior portion of the abdominal cord, containing the fifth and last abdominal ganglia, was examined in 6-micron serial sections. A mercuric chloride-acetic acid mixture provided most satisfactory fixation. Ester wax (Steedman,

1947), with increased paraffin content, proved an excellent embedding medium. Conventional staining techniques included: Mallory's triple stain, Masson's trichrome, Gomori's chromium-hematoxylin-phloxin (Gomori, 1941) and Holme's silver as modified by Batham and Pantin (1951). Desheathed specimens were fixed after physiological tests.

A series of cords was stained by Laidlaw's method as described by Krnjević (1954) after the fifth ganglion and one half of the connective between fifth and sixth ganglia had been desheathed. The living cord was placed in 0.5% silver nitrate and observed under strong illumination. One of this series was fixed, sectioned and counterstained with Mallory's (Fig. 3b).

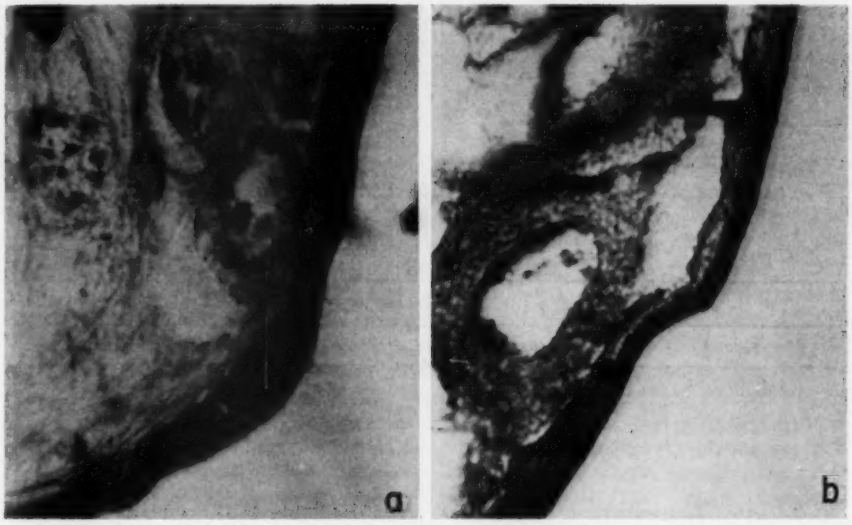

FIGURE 3. (a) Normally sheathed last abdominal ganglion stained with Masson's trichrome. Note outer homogeneous layer and inner nucleated granular layer of sheath. (b) Normally sheathed last abdominal ganglion after silver nitrate treatment. Note adherence of inner and and outer sheath layers in region of shrinkage. Note silver granules in sheath, absence of granules in interior of ganglion. Counterstained with Mallory's triple stain.

OBSERVATIONS

Osmotic changes

The intact, normally sheathed cord was not observed to swell or shrink and nervous conduction was unaffected when total solute concentration was reduced to the equivalent of 0.140 *M* NaCl by dilution or increased to 0.180 *M* by adding NaCl or sucrose to normal Hoyle's saline (0.156 *M*). Desheathing in the hypotonic (0.140 *M*) saline resulted in gross swelling and functional impairment. Synaptic conduction failed totally. Desheathing in the saline hypertonic to Hoyle's saline (0.180 *M*) resulted in no immediate structural or functional alternation.

High potassium saline

In Table I are listed typical times for total conduction block on perfusing a previously untreated cord with 0.180 *M* KCl or Hoyle's balanced saline containing 140 mM/L. K^+. Recovery was followed in K^+- free saline.

Blocking was more rapid in 0.180 *M* KCl. This could well be attributed to the absence of sodium and other ions rather than higher K^+ concentration. Desheathing obviously reduced recovery time as well as blocking time. The presence of the cut ends of small nerves (severed in dissecting the fourth ganglion) did not alter the blocking time appreciably.

TABLE I

Effect of potassium ions on impulse conduction in intact and desheathed cords

A. Intact segment

Solution	Blocking time	Recovery time	Type preparation
0.180 *M* K^+	12 min.	10 min.	Connective only
0.180 *M* K^+	15 min.	10 min.	Ganglion included
0.180 *M* K^+	18 min.	20 min.	Ganglion included
140 mM./L. K^+	30 min.	25 min.	Ganglion included
140 mM./L. K^+	22 min.	33 min.	Ganglion included

B. Test segment desheathed at B (see Fig. 1a)

Solution	Blocking time	Recovery time	Type preparation
0.180 *M* K^+	10 secs.	4 min.	Ganglion included
140 mM./L. K^+	60 secs.	2 min.	Ganglion included
140 mM./L. K^+	90 secs.	2 min.	Ganglion included

Repeated applications of high K^+ solutions to intact cord segments led to increasingly rapid block and delay in recovery. This effect was not seen in desheathed nerve.

Sodium-deficient saline

Substitution of sucrose for the sodium of the normal saline did not affect the normally sheathed segment in experiments continued several hours, although this caused conduction block in a stripped preparation within 30 seconds (Fig. 1b). Recovery was complete within two minutes in normal saline.

Acetylcholine

Treatment of the intact ganglion with extremely high concentrations of acetylcholine (10^{-2} *M*) did not alter synaptic responses (Fig. 2 a, b), as had been reported by Roeder (1948). However, effects of 10^{-2} *M* acetylcholine on synaptic function in the desheathed ganglion were usually rapid and pronounced. In two of a series of seventeen experiments, only a moderate decrease in synaptic response

was noted. In all others, within one to five minutes bursts of asynchronous action potentials were followed by synaptic depression and block. Figure 2 shows the rapid and easily reversible block of a ganglion desheathed in 10^{-2} *M* acetylcholine. The lowest effective concentration of acetylcholine was between 3 and 5×10^{-3} *M* in the uneserinized desheathed ganglion. After eserine, acetylcholine between 10^{-4} *M* and 10^{-3} *M* blocked the synapse. No effect on axonic conduction was noted.

Sheath structure

Since the foregoing experiments demonstrated the functional importance of the sheath, an attempt was made to examine its mechanical properties and structure.

It was extremely difficult to penetrate the sheath of the intact nerve cord with any object. Even a finely tapered capillary microelectrode merely dimpled the surface and broke. After desheathing, penetration of the cord and individual neurons presented no difficulty. During dissection, the sheath felt tough and elastic. As has been mentioned, in some dissections the sheath severely constricted the cord. The almost explosive bulging-out of nerve substance through a small hole made in the sheath when the preparation was immersed in hypotonic saline strikingly illustrated the mechanical resistance the sheath offers to swelling. Injected air bubbles were trapped beneath it. Histologically, the sheath was continuous and clearly double-layered. The outer, almost homogeneous layer, two to five micra in thickness, stained deeply with aniline blue. Occasional nuclei were evident in this layer, possibly representing fibroblasts. The inner layer consisted of granular squamous epithelial cells, one to three micra thick. The flattened nuclei of the inner layer were prominent in both cross and coronal sections (Fig. 3a). (The outer layer referred to above corresponds to the homogeneous, non-cellular, neural lamella described by Scharrer (1939) and Hoyle (1952), the inner layer to the thin, continuous cytoplasmic cylinder which Scharrer terms the perineurium and Hoyle terms the perilemma.) These layers were closely adherent to one another and pulled away together from the nerve substance when the preparation was desheathed or when shrinkage occurred in fixation (Fig. 3b). No other continuous structures lay external to these layers in our intact preparations. These two layers were always absent in areas functionally desheathed.

It should be mentioned that within the nerve cord itself, fibrous investments, almost capsular in appearance in silver-stained sections, but probably discontinuous, surrounded certain large cell bodies and groups of cell bodies. These fibrous investments may correspond to the glial elements described by Scharrer (1939). The giant axons were individually encased in a delicate (one micron in thickness) sheath-like structure throughout their length.

When a freshly-dissected, unfixed cord was observed during soaking in silver nitrate, the normally sheathed areas showed at first a fine network of black lines, then became stippled and blotched in appearance and finally almost uniformly black. The blackening was close to the surface and the deep black layer could be lifted off by desheathing. Desheathed cord soaked in silver nitrate did not blacken superficially, but individual neurons could be observed to stain black in these preparations. Sections of the intact last abdominal ganglion, treated while fresh with silver nitrate, showed black granules concentrated in the surface layer corresponding to

the sheath (Fig. 3b), while the desheathed fifth ganglion, after similar treatment, showed granules distributed throughout.

Discussion and Conclusions

The sheath surrounding the nerve cord of *Periplaneta* clearly limits diffusion of ions, as Hoyle (1953) showed in *Locusta*. However, in efficiency of sheath function as measured by total time required for block in excess potassium, the intact *Periplaneta* cord is more comparable to the amphibian sciatic nerve, which will block in 13–20 minutes in isotonic KCl (Lorente de Nó, 1947; Feng and Liu, 1949) than to *Locusta* nerve, which resists block as long as four hours in saline containing 140 mM potassium, provided the tracheal supply is undamaged. Hoyle pointed out the importance of adequate oxygenation in maintaining sheath function. It may be that in our preparation, since abdominal movements were severely restricted, oxygenation through the tracheal supply was insufficient to maintain sheath function although efforts were made to avoid all damage to the tracheal supply.

The retardation of potassium diffusion is a two-way function of the sheath, judging by the large decrease in recovery time as well as blocking time in desheathed cord. The cumulative effect of repeated high potassium solutions, as well as the appearance of the silver nitrate-treated cord (Fig. 3b), suggests that ions penetrate the sheath readily but are accumulated there. Shanes (1954) showed with tracer techniques that the sheath of the frog sciatic nerve is entirely responsible for retardation of ion diffusion outward as well as inward.

Desheathed *Periplaneta* cord is soon blocked by sodium deficiency, although conduction in the intact cord is unaffected for long periods in sodium-free saline. This, then, is direct evidence that insect neurons are similar to the neurons of other groups not only in potassium sensitivity but in their susceptibility to inactivation by sodium deficiency, and that it is the sheath which masks this susceptibility just as does the sheath which invests vertebrate nerve (Huxley and Stämpfli, 1951).

Although much pharmacological evidence suggests the cholinergic nature of the synapse between cercal nerves and giant fibers in the last abdominal ganglion of *Periplaneta*, not even very high acetylcholine concentrations affect the intact ganglion. This total lack of effect is clearly due to restriction of acetylcholine penetration by the sheath. However, effective concentrations of acetylcholine are high even in desheathed preparations so that the synaptic specificity of the acetylcholine action is still in question. (See Twarog and Roeder, 1957 for further data.) The sheath-like structure which invests the giant fibers may represent another barrier to ion diffusion, or the synaptic terminations of the cercal nerves may be otherwise "protected."

Water diffuses through the sheath quite readily, as is evident from the immediate swelling-out of nerve substance which occurs when a small portion of the sheath is removed while the cord is soaking in hypotonic saline. It is obvious that the physical restraint exerted by the sheath in preventing swelling limits total water uptake and preserves structural integrity. Lorente de Nó (1952), Shanes (1953) and Krnjević (1954) have emphasized the possibility that the mechanical rigidity of the vertebrate sheath may serve an osmoregulatory function. The fluctuations in hemolymph water in insects are often very great (Mellanby, 1939), and an osmoregulatory function of the sheath may be as important as its role in salt regulation.

The actual site of ion regulation by the sheath is in one or both of the two layers described above, which were also described by Hoyle (1952) in *Locusta*. Huxley and Stämpfli (1951) suggested that ion regulation by the frog sheath is a function of the innermost epithelial layer first described by Ranvier in 1876 rather than the loose connective tissue of the epineurium. Krnjević (1954) showed conclusively that this is true. Within the epineurium he described the two-layered perineurium: an inner continuous layer of squamous epithelium, 4–6 micra thick, with an external layer of comparatively undifferentiated connective tissue. In some regions of the sciatic nerve additional cellular layers were seen, but these were not continuous over the entire nerve. He succeeded in showing that the silver granules are most concentrated in the epithelial layer. The analogy in structure between the insect sheath and the frog perineurium is rather striking. It is likely that it is the squamous epithelial layer which fulfills the important regulatory function in the insect. Krnjević (1954) has discussed the importance of epithelial layers in regulation of diffusion through capillary walls and through the connective tissue sheaths which surround nervous structures in vertebrates. The inner, epithelial layer of the sheath may well bear some physiological and structural resemblance to the so-called blood-brain barrier.

It must be mentioned here that the desheathing technique, in addition to having utility as a method of studying this interesting ion-regulating structure itself, presents advantages in investigating insect nervous function. It has been employed by Twarog and Roeder (1957) in pharmacological studies to avoid total failure of drug penetration or insufficiently rapid penetration to the site of action. Perhaps more important is the fact that it makes possible routine exploration of the insect nervous system with microelectrode techniques. Resting cell membrane potentials of 50 to 70 MV have been easily measured and sustained. Cell action potentials have been obtained but no systematic study has yet been made.

Summary

1. A method is described for removing portions of the connective tissue sheath which invests the nervous system of the cockroach, making possible studies of sheath function and of basic physiological properties of the insect neuron.

2. This sheath restricts diffusion of potassium and sodium ions and of acetylcholine from the surrounding fluid to the interior of the cord.

3. The ability of the sheath to restrict swelling suggests a possible osmoregulatory function.

4. The functional sheath consists of an inner continuous squamous epithelial layer and an outer, almost homogeneous connective tissue layer.

5. This study confirms the conclusions of Hoyle (1952, 1953) with respect to ion regulation by the insect sheath, and indicates close functional and structural parallels in the sheaths investing insect and vertebrate nervous tissue as well as in the basic properties of the nervous tissues of insects and vertebrates.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1956

ABSTRACTS OF SEMINAR PAPERS

JULY 10, 1956

Amphibian yolk: chemistry and ultrastructure. PAUL R. GROSS.

The amphibian yolk platelet is a complex structure, both chemically and physically. Intact washed platelets contain protein, lipid, non-protein nitrogen, organic non-protein phosphorus, and pentose, probably in PNA. DNA is absent, as shown by a negative diphenylamine reaction. The protein of the platelets is made up of at least three macromolecular species, probably more. Sedimentation experiments at a variety of pH and ionic strength levels show that the main component, vitellin, accounts for more than 85% of the protein, while the remainder is in the form of two apparently polydisperse populations of particles. The vitellin sediments at about 12 S.

Further kinetic studies of the reaction in which the yolk platelets are lysed by low concentrations of Ca^{++} strengthen the hypothesis that this reaction is mediated by a Ca-activated enzyme system which resides within the platelet. As the concentration of Ca is lowered, the dissolution reaction undergoes a change of kinetic order from one to zero. Such a situation would not be expected were the solubilization a result of simple salting in, as is indeed the case in uniunivalent salts at ionic strengths greater than 0.4. The Ca-dissolution reaction also has an optimum over-all ionic strength which lies far below the minimum value for dissolution by uniunivalent salts.

The platelets are solids in water, and can be broken with microneedles into insoluble fragments. As the ionic strength is raised, the platelets are seen to swell and assume a more isodiametric shape, and at this point, preceding complete disruption, they are seen to be surrounded by a membrane. We have been able to photograph this "ghost"-like structure with the electron microscope.

Amphibian yolk: the phosphoprotein phosphatase system. SYLVAN NASS.

The "PPP'ase" activity of yolk platelets isolated from ovarian frog eggs was studied under a variety of experimental conditions. The pH optimum for platelets incubated in NaCl of ionic strength between 0.1 and 0.4 was 4.9. The temperature optimum for 90-minute incubations was 45° C. In systems to which $CaCl_2$ ($\mu = 0.25$) had been added, the pH optimum was 4.3, while the temperature optimum was at 37° C. Relative to the uniuvalent salt, $CaCl_2$ acts in these systems as an inhibitor of P release. The Ca^{++} solubilizes the platelets, but no correlation between this process and P release was found.

Differences in PPP'ase activity between platelets incubated in NaCl at $\mu = 0.1$ and $\mu = 0.4$ can be directly related to the structural integrity of the platelet. At low ionic strength, the platelets usually remain intact during the incubation periods at the chosen temperatures and pH. Increase in ionic strength causes rapid dissolution of the platelets. Variations in temperature, pH, and time of incubation also determine the solubility of these structures. In all systems in which solubilization occurs (no Ca present), there is an increase in P release over that in control systems with platelets intact.

Kinetic studies show that the P release from intact platelets at any time is approximately half of that from platelets solubilized at the outset of an experiment. If then the intact platelets

are dissolved by any means other than adding Ca^{++}, a sharp rise in the ($P_{inorg.}$) is obtained, bringing this value to that in the system solubilized at zero time. This may be interpreted in terms of a membrane-like diffusion barrier investing the platelet which retards release into the bulk phase of the hydrolyzed phosphate.

The relation of gonad hormones to x-irradiation sensitivity in mice. JOAN WOLFF AND ROBERTS RUGH.

A single injection of estradiol benzoate was made I.P. 10 days before whole body x-irradiation exposure to an LD/50/30 day level of 49 normal intact male CF_1 mice and 41 mice which had been castrated by surgical excision. Fifty other castrates and fifty intact mice, all of the same age, received no injections. Lethality data were collected for a period of 30 days following the irradiation. When the percentage survival is plotted against days post-irradiation, it is found that the intact male controls, which received no hormone injection, all died by the 17th day. At 30 days post-irradiation some 38% of the castrated males were still alive, indicating an adverse effect of the presence of the male hormone, testosterone, in the controls. Maximum survival was found in the castrated males which had also been injected with estradiol benzoate. The normal intact males which had been injected with this hormone showed a survival value almost as good, namely 63.3%. Thus the injected female hormone must have counteracted almost entirely the adverse effects of the male hormone. The differential in survival of male and female mice, when exposed to whole body penetrating x-irradiation, seems to be due in part to the gonadal hormones present. Intact males, with the normal quota of testosterone, have less survival value than do castrate males. Further, castrate males injected with the female hormone, eat radiole benzoate, exhibit maximum survival, approaching that of the intact female.

GENERAL SCIENTIFIC MEETINGS

AUGUST 20–22, 1956

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read By Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Experimental hypothermia and carbon dioxide production in the white rat. C. LLOYD CLAFF, FREDERICK N. SUDAK AND NAOMI R. STONE.

A technique was described whereby carbon dioxide production could be measured in conscious rats utilizing barium hydroxide, N-butyl alcohol and thymolphthaline indicator. The efficacy of this method was tested with a carbon dioxide generator. Carbon dioxide up to seven times the output of a normal rat was generated, and 100% of this amount was collected.

Twelve-hour fasted male white rats were injected with Thorazine; 2,4-Dichlorophenoxyacetic acid; and a combination of the two drugs. Studies at room temperature (24° C.) revealed that a combination of Thorazine and "2,4 D." decreased the rectal temperature of a white rat from 36.3° C. to 31.8° C. with a diminution in carbon dioxide production of 30% in five hours. This was in marked contrast to the temperature-carbon dioxide relationship in animals which received only Thorazine. The rectal temperatures of these rats were reduced 6.0° C. in seven hours with an increase of 33% in carbon dioxide output.

One and one half hours after injection, animals were subjected to 2.0° C. Twenty minutes later, CO_2 and temperature measurements were taken. Normal rats increased their CO_2 output 136%, and maintained normal body temperatures. Animals receiving "2,4 D." and Thorazine in combination showed no significant change from normal output in their carbon dioxide, and a decrease in rectal temperature of 6.5° C. Thorazine-injected rats increased their CO_2 output

91% and rectal temperature dropped 5.5° C. Animals injected with "2,4 D." could only increase CO_2 output to 54%. A drop of 3.0° C. in rectal temperature occurred. All animals survived, and normal behavior patterns were observed within 24 hours.

The possibility of inducing hypothermia by interfering with the temperature regulating mechanism of a homoeothermic animal by means of drugs was discussed.

Sperm entry in Hydroides hexagonus (Annelida) and Saccoglossus kowalevskii (Enteropneusta). ARTHUR L. COLWIN, LAURA HUNTER COLWIN AND DELBERT E. PHILPOTT.

The authors have demonstrated previously (1954, 1955, 1956) that in a number of species the acrosome filament of the spermatozoon penetrates into the egg proper. The present species were selected for electron microscope studies because their eggs are essentially jellyless and their spermatozoa make direct contact with the egg membranes. Moderate polyspermy was induced in order to increase the chances of finding examples of sperm-egg association in thin sections. It is not established whether or not the spermatozoa here described were the specific ones which initiated the activation of the eggs studied.

In Hydroides, electron microscope photographs show that at the earliest contact between spermatozoon and egg the acrosome touches the thin, hexagonally patterned, layer which surrounds the much thicker vitelline membrane. Subsequently this thin outer layer seemingly rises up around the sperm head, even before a major part of the sperm head has entered the membrane. The vitelline membrane, too, appears to rise up around the sperm head, thickening in this vicinity. Near the acrosomal region of spermatozoa which appear to have breached the vitelline membrane completely, the perivitelline space is almost twice as wide as elsewhere. The above described effects suggest some lytic action stemming from the spermatozoon.

In Saccoglossus, electron microscope photographs show that acrosome filaments penetrating the outer and vitelline membranes are spiral in shape. The outer egg membrane is pitted wherever spermatozoa adjoin it. These pits are interpreted as resulting from erosion of the membrane by a lysin from the spermatozoon, demonstrating this lytic action at the level of the individual spermatozoon. The authors previously reported (1954) such pits in relation to sperm entry in living material as seen by light microscopy.

Electron microscope studies of the egg surfaces and membranes of Hydroides hexagonus (Annelida) and Saccoglossus kowalevskii (Enteropneusta). LAURA HUNTER COLWIN, ARTHUR L. COLWIN AND D. E. PHILPOTT.

With light microscopy, the vitelline membrane of living Hydroides eggs appears thick and uniform. Early in fertilization this membrane changes little and fails to elevate rapidly. The perivitelline space does not enlarge rapidly. With electron microscopy, sections of unfertilized eggs show a wide felt-like vitelline membrane closely surrounded by a single layer of very evenly spaced minute bodies linked in hexagonal pattern by fine threads. Similar threads link the layer to the membrane. Larger, fewer, microvilli project regularly from the egg proper, deeply penetrating the vitelline membrane. In cross section, at least the broader proximal part of the microvillus shows a deeply staining periphery encircling a central unstained region. No well-defined *layer* of cortical granules is seen. No great change is observed following fertilization. However, a narrow perivitelline space does develop; across this the microvilli project into the membrane for some time.

Light microscopy shows two membranes surrounding the living unfertilized Saccoglossus egg. Sections reveal a well-defined cortical granular layer. At fertilization both membranes elevate rapidly. The inner one (fertilization membrane) successively thickens, apparently receives additional material from the cortical layer, and becomes compressed. Simultanously, the egg surface forms short-lived irregular protuberances, the cortical layer disintegrates, hyaline granules and threads erupt from the egg into the rapidly enlarging perivitelline space. In living eggs much of this material becomes invisible, though a border of hyaline granules persists close to the egg. Sections show a reticulum in the perivitelline space. Electron microscopy verifies the above observations, shows release of cortical granular material, and also reveals

stout microvilli between the egg and the vitelline membrane but not penetrating the latter. Upon fertilization these microvilli elongate, apparently detaching from the membrane; their ultimate fate is unknown. Cross sections of elongated microvilli show a well-defined circular central structure apparently surrounded by eight dot-like bodies.

Ionic regulation in the fiddler crabs, Uca pugnax and U. pugilator. JAMES W. GREEN, MARY HARSCH, LLOYD BARR AND C. L. PROSSER.

Three species of *Uca* (*pugnax, minax* and *pugilator*) like *Pachygrapsus* but unlike *Carcinus* and *Callinectes* were found to have lower concentrations of Na in urine when adapted to 175% than to 100% S.W. In *U. pugnax* and *pugilator,* the two species selected for more detailed study, the total osmotic concentrations of both urine and serum were slightly higher in crabs from the 175% S.W. than those from the 100%. Analyses of urine and serum from both groups for Na, Mg, Ca, K, Cl and SO_4 showed a cation deficit in the urine of about 20% which was found to be largely NH_4. Although the serum Na values of both groups were approximately the same there was 30 to 40% less Na in the urine of crabs in the 175% S.W. and the cation deficit thus incurred was made up by the excretion of about 3 times as much Mg as was found in the urine of the 100% crabs. When both groups were placed in S.W. containing Na^{24} the isotope was found to enter at essentially the same rate in each group. Tissue analyses of Na^{24}-labeled crabs indicated that Na was not being stored. When Na^{24}-labeled crabs were washed in S.W. without isotope, placed in dry finger bowls for short periods and samples of gill fluids analyzed, it was found that crabs from the 175% S.W. contained a higher proportion of Na^{24} in their gill fluids than the 100% group. This was interpreted to mean that while both groups lost Na through the gills the crabs in 175% S.W. excreted more by this route, thus accounting for the lowered urine Na values found.

Electron microscopy of the mitotic apparatus in dividing Arbacia eggs. PAUL R. GROSS, DELBERT E. PHILPOTT AND SYLVAN NASS.

In the course of an investigation of the mechanisms of sol-gel transformations in dividing cells, it became a matter of interest to determine the nature of the material which constitutes the mitotic spindle. Considerable data concerning this problem have been reported from Mazia's laboratory, but good, high-resolution electron micrographs of the achromatic figure are lacking. We have devised a new method for observing this structure with the electron microscope. The basis of the procedure is the observation of Mazia and co-workers that dividing cells can be killed with cold 30% ethanol without too extensive denaturation of cytoplasmic proteins and without dissolution of the mitotic figure.

The cells are fixed in the desired stage of mitosis with 30% ethanol at $-10°$ C. for about 30 minutes, then transferred to 30% ethanol containing 1% osmium tetroxide at $-10°$ C. After fifteen minutes in the cold osmic-ethanol, the suspension is removed to room temperature and permitted to warm slowly. After about fifteen minutes more, the eggs are quickly sedimented down and washed with 50% alcohol, thence run through a series of alcohols for dehydration. The cells are embedded as usual in a mixture of methyl and n-butyl methacrylates, and sections are cut on a microtome designed for ultra-thin sectioning.

Using this procedure, we have been able to observe in detail the structure *in situ* of the metaphase spindle and of the chromosomes at the metaphase plate. The spindle appears to be a gel composed of elementary particles of widely varying diameter, with a mean value near 400 Å. At high magnifications, these particles are seen to be themselves composites of somewhat asymmetric particles of diameter ca. 30–50 Å.

Participation by actin in actomyosin contraction. TERU HAYASHI, RAJA ROSENBLUTH, PETER SATIR AND MICHAEL VOZICK.

Any mechanism of muscular contraction at the molecular level must take into consideration the behavior of the muscle proteins, actin and myosin. The results of recent *in vitro* experiments have been interpreted to indicate that only myosin is the contractile protein, with

actin playing an associated role, but not directly involved in the configurational changes resulting in contraction. Therefore, a series of experiments was done to test whether myosin alone was capable of contraction, without actin.

Purified myosin was prepared, and by viscosity tests and solubility tests shown to contain less than 0.1% actin. Pellicular fibers formed of this material at pH 7.0, and 0.05 *M* KCl, failed to develop tension upon addition of ATP, either at pH 7.6 or 9.0. Reconstituted actomyosin combining graded amounts of actin and myosin, developed graded tensions at pH 7.6, whereas at pH 9.0, there was no contraction. The graded tensions developed corresponded roughly to the "activity" values obtained from viscosity tests, which are in turn proportional to the amount of actin present. Isotonic contractions corroborated the findings obtained from experiments done under isometric conditions. Myosin fibers, formed at low temperatures with ATP also applied at low temperature, failed to develop tension when the temperature was raised, whereas actomyosin fibers treated similarly readily developed tension with the increase in temperature.

Implications as to the role of actin will be discussed. It is concluded that the contraction involves actin specificially and directly, and that myosin alone is not capable of contraction.

An interpretation of the action of certain chemical agents used in cancer therapy.[1] L. V. Heilbrunn and Walter L. Wilson.

In spite of the vast literature on the chemotherapy of cancer, but little is known as to why various agents act as they do, and there has scarcely been even a hypothesis proposed as to why some agents which have a carcinostatic action may also be carcinogenic. We have studied the effect of nitrogen mustard, Nitromin, 6-mercaptopurine and urethane on eggs of the marine worm Chaetopterus. All of these substances have been used in the treatment of cancer. In dilute solution, all of them prevent cell division, and all of them act by suppressing the mitotic gelation. In more concentrated solutions, nitrogen mustard, Nitromin and urethane have the opposite effect, that is to say they tend to induce protoplasmic clotting. Thus they might well act to initiate mitosis, for the initiation of mitosis is caused primarily by agents which cause protoplasmic clotting. Indeed it can be shown that solutions of urethane do actually induce mitosis in Chaetopterus eggs. These facts are in line with the colloidal theories discussed in some detail in Heilbrunn's Dynamics of Living Protoplasm, published earlier this year by the Academic Press. Fractions of cow ovary extracts obtained by cold alcohol fractionation were also tested on Chaetopterus eggs. Fractions which prevent mitotic gelation act as rather powerful carcinostatic agents. This has been established by other members of our group who are now working with Ehrlich ascites tumors in mice.

A striking behavioral change leading to the formation of extensive aggregations in a population of Nassarius obsoletus.[2] Charles E. Jenner.

The distribution of *Nassarius obsoletus* in a given locality frequently presents problems of considerable ecological interest. Snails are sometimes absent from apparently favorable areas, although present in enormous numbers in adjacent but similar areas. The factors underlying this extreme patchiness in distribution are not readily apparent.

The problem was illustrated by a striking change in distribution pattern which occurred in a population of snails in Barnstable Harbor during the summer of 1956. The area under observation was an extensive mud-sand flat, approximately 200 × 800 yards, exposed only at low tide. In a well-populated area a reference zone was staked, marking 10 adjacent quadrats, 10 × 10 yards. During visits on June 19, July 1, 7, 15 and 22, snails were present in great numbers but were widely distributed over most of the flat. Observations during a brief visit on July 30 were hampered because the flat was not exposed at the time. It was apparent, however, that a definite change in snail distribution had taken place since the last visit; snails could be

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

found in only one of the 10 quadrats. During the next observation (August 4), snails were not to be found over most of the flat and none were present in the marked area. The snails occurred chiefly in massive aggregations; in many areas they literally covered the ground, in some cases two or three layers deep. No snails were within 180 feet of the marked area, indicating that most had traveled more than this distance during the 13 days since the July 22 observation.

The functional significance of such mass migration and aggregation is not readily understandable. In the formation of the aggregations it seems clear that the animals were not responding independently to inanimate factors. Some type of interaction between snails must have been involved.

The timing of reproductive cessation in geographically separated populations of Nassarius obsoletus.[1] CHARLES E. JENNER.

The termination of seasonal reproductive activity was followed in populations of *Nassarius obsoletus* from Beaufort, N. C.; Great Pond, Mass. (south Cape Cod); and Barnstable Harbor, Mass. (north Cape Cod) during the spring and summer of 1956. In males of *N. obsoletus* the copulatory organ is resorbed at the end of the reproductive season (Jenner and Chamberlain, 1955), and the state of reproductive activity was judged by recording the per cent of unparasitized males bearing this structure. The per cent of unparasitized females having a formed egg case in the reproductive duct was also recorded.

For a given population cessation was found to be abrupt, with the major part of the transition period being only two to three weeks in duration. Curves for males and females were nearly superimposed. Reproductive activity was essentially completed for Beaufort snails by the end of May, for Great Pond by mid-July and for Barnstable by the latter part of August. The displacement of the curves for the different populations seems to be related to temperature, and agrees nicely with the known distribution of temperature along the Atlantic coast during the spring and summer months (Parr, 1933). The difference in timing, for example, between the north and south sides of Cape Cod (a latitudinal difference of 12 miles) was about as great as between south Cape Cod and Beaufort (latitudinal difference of over 500 miles). A May 17 collection from Mayport, Florida, indicated a timing of reproductive cessation similar to that of the Beaufort snails. Limited observations on snails from Boothbay Harbor, Maine, showed these snails to be two to three weeks later in their cycle than snails from Barnstable.

The method employed allowed a high degree of precision in the description of this seasonal event.

Absence of membrane potential in presence of asymmetrical ion distribution in the Fundulus egg. C. Y. KAO.

The accumulation of ions by biological membranes has generally been assumed to be accompanied by an electrical potential difference. In almost all excitable tissues at rest and in the egg of *Asterias forbesii*, the membrane potential approaches the value obtainable on the basis of the ratio of $[K^+]$ on the two sides of the membrane. In the *Fundulus* egg, no membrane potential can be measured, in either the unactivated or activated state, across the plasma membrane, although a high membrane resistance is present. By means of flame spectrophotometry, potassium and sodium contents have been determined, using homogenized or aspirated egg material. K is present in 105 *mM*/liter of egg material in the unactivated egg, and 117 *mM* in the activated egg, whereas Na occurs in ca. 50 *mM*. The values for the corresponding ions in sea water are 10 *mM* and 452 *mM*, respectively. In addition to a lack of membrane potential, membrane resistance of activated egg is not materially affected by immersion in isotonic chloride solutions of Na^+, K^+ (540 *mM* each), Ca^{++}, and Mg^{++} (370 *mM* each) for up to 10 min. The discordance between the concentrations of the two major cations and the electrical properties of the egg plasma membrane should preclude generalizations with respect to the origin of membrane potentials in biological material.

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

Electron microscopic observations on the development of the chorion of Fundulus. NORMAN E. KEMP AND MARGARET D. ALLEN.

Pieces of ovaries containing developing oocytes were fixed in buffered (pH 7.4) 1% osmic acid in artificial sea water, embedded in methacrylate and sectioned with a Porter-Blum microtome set to cut at 0.025 μ. Sections reveal that cells of the follicular epithelium pull away from the oocyte as deposition of the zona radiata begins. The zona is a fibrous membrane perforated by so-called pore canals from the time of its first appearance. It thickens chiefly by the addition of new material on its inner surface. The pore canals, which account for the striated appearance of the zona radiata with the light microsocpe, contain microvilli, spirally coiled in older oocytes, extending from the surface of the oocyte outward to the subfollicular space. Branching protoplasmic processes of the epithelial cells are in close contact with, or possibly continuous with, the microvilli in the subfollicular space. Among the follicular cell processes are extracellular strands or filaments. The chorion of the ovulated egg is composed of (1) an internal portion, the chorion internum (Shanklin and Armstrong, 1952), consisting of lamellae arranged in a herringbone pattern, and (2) an outer portion, the chorion externum, consisting of tangentially oriented filaments in an adhesive matrix. The chorion internum is the transformed zona radiata of the oocyte; and the chorion externum is derived from the filaments and intercellular matrix of the follicular cell processes. We conclude that the chorion is anatomically a vitelline membrane (lamellated portion) combined with a true chorion (filamentous portion).

The nature of the metal-to-protein bond in hemerythrin. IRVING M. KLOTZ AND THEMIS A. KLOTZ.[1]

Hemerythrin, an oxygen-carrying pigment found in sipunculid worms, contains iron at its active sites but is devoid of porphyrin groups. Thus the metal seems to be attached directly to the protein. On a stoichiometric basis each active site, holding one O_2 molecule, corresponds to 2.4 Fe atoms.

Displacement experiments with other metal ions suggested that the iron is attached to sulfide groups of the protein. Titrations for mercaptan groups were carried out, therefore, with silver ion. The color of the oxyhemerythrin disappeared as silver was added and at the amperometric end point 2 Ag had been taken up for each 2.4 Fe in the protein. It would seem, therefore, that 2 of each 2.4 irons are attached to mercaptan side chains. Confirmation that the silver really displaces iron from a sulfide bond was obtained from quantitative studies of the reaction of oxyhemerythrin with a disulfide dye. The dye should react specifically with protein —SH groups; colorimetric titration agreed well with the silver amperometric titration.

These observations suggest that each active site of hemerythrin contains two iron atoms attached to the protein through cysteine side chains, the iron atoms holding an O_2 molecule between them. This configuration would also conform with previous results on the valence changes in the metal during the oxygenation process as well as with the new observation that approximately one-half as much mercury-(II) as silver-(I) combines with hemerythrin in amperometric titrations.

The molecular weight of hemoglobin from Petromyzon marinus. P. GALEN LENHERT,[2] WARNER E. LOVE AND FRANCIS D. CARLSON.

The present study was undertaken as a preliminary to a proposed x-ray diffraction analysis of this substance. An accurate molecular weight of Petromyzon hemoglobin was desired before attempting crystallization.

The sedimentation coefficient corrected to water at 20° C. was found to be 1.9 S units. A molecular weight of 23,600 was calculated from data taken during the approach to sedimenta-

[1] This investigation was supported in part by a research grant, RG-4134, from the National Institutes of Health, Public Health Service.

[2] National Science Foundation Fellow 1955–56.

tion equilibrium by the method of Archibald as modified by Klainer and Kegeles. A value of 0.751 given by Svedberg and Eriksson-Quensel as the partial specific volume of Petromyzon hemoglobin was used in this calculation. This figure must be considered approximate due to our inability to obtain optimal photographic conditions.

Iron was determined by Lorber's sulfosalisylic acid method. The values obtained, using an extinction coefficient for the iron-sulfosalisylic acid complex of 0.101 per microgram of iron per mililiter, indicate one mole of iron for every 17,800 grams of protein. Heme was measured as the reduced pyridine hemochromogen, giving a value of 18,600 grams of protein per mole of heme.

These results in conjunction with the sedimentation equilibrium molecular weight given above support the conclusion that there is one heme group per molecule of hemoglobin. The molecular weight which results from these data is $18{,}200 \pm 400$. This result of approximately 20,000 is in agreement with Svedberg's values for other cyclostome hemoglobins. Attempts are now being made to crystallize this protein.

Hyaline polymer of the fertilized egg of Arbacia punctulata. ARTHUR K. PARPART AND JULIEN CAGLE.

The hyaline layer which forms during the first 5 to 10 minutes after fertilization of the egg of *Arbacia punctulata* is the resultant of a polymerization of a polysaccharide probably released as a monomer upon the explosive breakdown of the cortical granules. The hyaline layer can be depolymerized completely and rapidly, 30 seconds, in isosmotic solutions of non-penetrating non-electrolytes (*e.g.*, glucose, xylose, erythritol, glycerol), and partly depolymerized in the presence of (a) 0.5 *M* NaCl, (b) 0.5 *M* NaCl plus 0.01 *M* $CaCl_2$, and (c) 1.0 *M* glucose plus either 0.1 *M* guanidine HCl or 0.001 *M* $CuCl_2$. Since guanidine and copper are active protein precipitants it is suggested that the hyaline layer is primarily composed of a polymerized polysaccharide which can be readily depolymerized. Upon depolymerization this polysaccharide exerts an osmotic pressure equivalent to ca. 5% egg albumin, which draws water into the perivitelline space. A depolymerized hyaline layer can be repolymerized by means of magnesium ions (0.005 *M* to 0.04 *M*) dissolved in isosmotic glucose, and an optically refractile and dense hyaline layer is re-formed. Upon repolymerization the colloid osmotic pressure in the perivitelline space decreases.

Magnifying the invisible. DELBERT E. PHILPOTT AND GEORGE G. LOWER.

A 16 mm. color movie has been made to show the basic techniques used in electron microscopy. An overall view of the R.C.A. Model EMU-2B and the North American Phillips Company Model EM-100 is shown. Specimen grids are prepared, and the complete process of a preparation of liquid suspension of virus is demonstrated. The shadow casting machine and technique are illustrated. To show how living tissues are prepared for the electron microscope, a frog heart is taken through the steps of fixation, embedding, and sectioning. Actual operation of the microscope is also shown. Various pictures of different tissues and specimens taken with the microscope are shown to demonstrate the adaptability of the electron microscope to a variety of research problems. A wave-length chart shows the difference between light and electrons as a source for microscopy, and a comparison of magnification sizes is demonstrated.

This film is meant to be an introduction to electron microscopy for the layman, to clarify to those who have never had the occasion to work with one, the basic principles and techniques used.

Sodium ion exchange in Ulva lactuca. GEORGE T. SCOTT, ROBERT DEVOE AND GARY CRAVEN.

Sodium ion was observed to exhibit an unexpectedly high rate of turn-over between the cells of *Ulva lactuca* and the surrounding sea water. This alga is a very favorable organism for the study of ion exchange since it consists of but two layers of cells. Furthermore, extracellular fluid with contained electrolytes can be removed by the combination of a thirty-second

rinse in isotonic sucrose and a triple blotting technique in absorbent tissue. Previously published data indicate that sodium and potassium ions diffuse into isotonic sucrose from the extra-cellular phases within three seconds.

The exchange experiments involved placing the alga for varying lengths of time in sea water containing 0.5 to 1.0 millicurie of Na^{24} per liter followed by the thirty-second sucrose rinse and the triple blotting procedure. The samples of algae consisted of small discs about two inches in diameter cut from a single large frond. Activities were determined, following which the samples were wet ashed and analyzed for sodium by flame photometry; specific activities were then calculated. Time course curves reveal that at twenty degrees the specific activities of the algae had reached equality with that of the sea water within ten seconds. At one degree exchange was nearly complete in fifteen seconds. Experiments done at twenty degrees, in which the sea water contained 1×10^{-3} *M* phenylurethane in one case, and in another 5×10^{-4} uranyl nitrate at pH 4.8, all in addition to the Na^{24}, showed a slight lessening of the exchange rate, the significance of which is in doubt. Uranyl ion has been shown to be preferentially surface adsorbed.

The data suggest, because of the very high rate of sodium turnover, an exchange of sodium ion between the surface of peripheral zone of the cell and the medium; this exchange probably does not directly involve an active metabolic pump *per se*.

Studies on parasites of the green crab, Carcinides maenas. HORACE W. STUNKARD.

Since *Carcinides maenas* is a serious predator of *Mya arenaria*, the U. S. Fish and Wildlife Service is interested in determining whether or not its parasites can serve as possible means of biological control. It has long been known that *C. maenas* in the Woods Hole area is infected by an undetermined, encysted metacercaria and experiments have been conducted to discover its identity, life-history and biology. Metacercariae were excysted to study their morphology and cysts were fed to recently hatched, uninfected birds, *Sterna hirundo* and *Larus argentatus*. Large numbers of worms were recovered, including all stages from juvenile to fully mature specimens. The structure of the metacercariae suggested that they may be specifically identical with a minute, stylet-bearing cercaria which occurs in *Littorina obtusata*, *Littorina saxatilis*, and rarely in *Littorina littorea*. Small green crabs exposed to these cercariae became heavily infected; enormous numbers of worms entered the tissues and developed to metacercariae, identical with those of the natural infections mentioned above. Small crabs exposed continuously with six to eight infected snails died in ten to twenty days, and on dissection yielded thousands of larvae. The stages in the life-cycle of the parasite agree with descriptions by European investigators of corresponding stages: the metacercariae with metacercariae from *C. maenas*, described but not named by M'Intosh (1865); the adults with *Microphallus similis* from Swedish gulls, described and named by Jägerskiöld (1900); and the cercariae with *Cercaria ubiquita* Lebour, 1907. The identity of these worms and their relation as stages in the life-cycle of a single species is predicated. It is possible that the parasites and their intermediate hosts are introduced species.

Conduction velocity in the giant axon of the squid (Loligo pealii) in D_2O. ROGER E. THIES [1] AND FRANCIS D. CARLSON.

A partially cleaned axon was threaded into a glass chamber with a volume approximately thirty times that of the treated region of the nerve. A few centimeters of the nerve were treated with a D_2O sea water (pH 8) made by evaporating artificial sea water to dryness and bringing the residue up to volume with 99.5% D_2O. A constant temperature bath maintained the preparation at $17.0 \pm 0.5°$ C. The stimulus was applied to an untreated region. The diphasic action potential was recorded within each end of the treated region, and both action potentials were displayed on the same single sweep of an oscilloscope. Measurement of the distance between the peaks of the action potentials gave the value of the conduction time to within $\pm 2\%$.

[1] National Science Foundation Fellow, 1956–57.

In eight experiments with five nerves the conduction velocity decreased by $19 \pm 3\%$ after treatment with the D_2O solution. This agrees with the finding of Garby and Nordquist on similarly treated frog sciatic nerve. Recovery in ordinary water solutions was to within $95 \pm 4\%$ of the original value. Both the initial decrease in velocity and the recovery were complete within three to five minutes. Lower concentrations of D_2O produced smaller decreases in the conduction velocity, the dependence being approximately linear. The effect is not due to small pH differences, since between pH 6.1 and 9.7 the conduction velocity remained unchanged to within $\pm 3\%$. The D_2O produced no detectable change in the diameter of the axon.

The small magnitude of the effect suggests that D_2O reduces conduction velocity because its viscosity is 1.23 times that of ordinary water, and ionic mobilities are correspondingly reduced. Further experiments are required to substantiate this hypothesis.

Change in rate of release of K^{42} upon fertilization in eggs of Arbacia punctulata. ALBERT TYLER [1] AND ALBERTO MONROY.[2]

From previous experiments demonstrating the existence of an electrical potential difference across the surface of echinoderm eggs, a rapid decrease followed by an increase of this potential upon fertilization, reversible depolarization by addition of K^+ externally, and a much greater concentration of K^+ inside the egg than in the surrounding sea water, it appeared that the process of fertilization has features in common with the stimulation of other excitable tissue such as muscle and nerve.

This leads to the expectation that there should be an increased rate of exchange of K^+ between the inside and outside of the egg upon fertilization.

By loading eggs of Arbacia with K^{42}, washing them, fertilizing aliquots and determining radioactivity of the supernatant, it has now been found that the rate of release of the K^{42} increases very markedly (1.5 to 3 X) upon fertilization. The results of seven experiments were consistent in showing that the increased rate of release starts within 1½ to 2 minutes after fertilization. There is also consistently a reduction of rate during the 5th to 8th minute, followed again by a rise.

The reversible replacement of potassium by rubidium ion in Ulva lactuca. ROBERT DEVOE, GEORGE T. SCOTT AND GARY CRAVEN.

Pieces cut from fronds of *Ulva lactuca* were placed in an artificial sea water containing rubidium instead of potassium ion, and samples harvested in triplicate at various times. After ninety-six hours the remaining pieces were placed in running sea water and samples harvested up to one hundred and twenty hours more. All samples were rinsed in isotonic sucrose solution and triple-blotted with absorbent tissue to remove all extracellular electrolytes, then ashed and analyzed for potassium, rubidium and sodium ion by flame photometry.

The uptake of rubidium was rapid and complete with four hours, the time the first samples were taken, and amounted to 65% of the original potassium present. The potassium ion concentration, after an initial drop to 40% of its original value, continued to decrease slowly in a manner parallel to the potassium ion concentration in the control. After ninety-six hours, when the samples were placed in running sea water, the gain in potassium and the loss of rubidium were rapid for the first ten hours, then more gradual. After one hundred and twenty hours in running sea water, the potassium ion concentration had practically reached that of the control, but the rubidium ion concentration had dropped to only 30% of its highest value. Sodium ion concentration was relatively constant during the course of the experiment.

Ulva lactuca does not show a time lag due to adaptation to rubidium as do some other organisms. It does photosynthesize when containing rubidium, and will form and discharge a germinal ridge.

[1] Supported by research grant C-2302 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Supported by a grant from Consiglio Nazionale delle Ricerche.

PAPERS READ BY TITLE

Observations on autotomy in the starfish, Asterias forbesi. JOHN MAXWELL ANDERSON.

Autotomy of rays in *Asterias* has excited the interest of many investigators but has never been adequately analyzed, apparently because attention has been focussed chiefly on subsequent processes of regeneration. Autotomy normally follows severe injury to a ray and can be artificially induced by application of electric current (8–16 V DC). As King (1898) reported, separation always occurs at one side or the other of a specific pair of ambulacral ossicles and proceeds rapidly at this level around the body-wall. The logical supposition that separation involves muscular contraction is verified by the fact that it is prevented by treatment of the animal with isotonic $MgCl_2$ solution. Under this narcosis, the animal can be subjected to radical operations, such as complete removal of its aboral body-wall; replacement in sea water and recovery from narcosis do not then evoke spontaneous autotomy. Electrically induced breakage in the floor of a ray thus exposed involves a sudden release of attachments binding the 5th pair of ambulacral ossicles to its neighbors; these ossicles, bound together by transverse muscular and collagenous fibers and bearing a pair of tube feet, can be lifted out with forceps after being thus released. Sections of these elements reveal that transverse connections are intact, but that the longitudinal muscle bands and connective tissues have been torn across. Initiation of autotomy does not necessarily depend upon this separation of ambulacral elements; it can be induced by electrical stimulation in the isolated aboral body-wall, where softening and tearing of muscular and connective tissues occur along a predetermined line. This cannot be regarded as simply a level of physical weakness, as attempts forcibly to break off a ray do not result in separation at this point. Serial sections of rays have shown no recognizable structural peculiarities of skeletal, muscular, or nervous elements in the region of the breaking-joint.

The innervation of muscle fibers in the extrinsic stomach-retractor strands of the starfish, Asterias forbesi. JOHN MAXWELL ANDERSON.

The extrinsic retractor strands are muscular bands extending in pairs from fibrous nodules on the outer wall of the cardiac stomach to attachments on the ambulacral ossicles in the floor of each ray. Their position raises a question as to the source of innervation for their muscular elements, whether by fiber tracts originating in the subepithelial nerve plexus layer of the gut wall or by pathways originating in the radial nerve cord of the ray. Vital staining procedures, utilizing leucomethylene blue, demonstrate that several groups of neurons traverse the mesentery-like sheets binding the strands to the floor of the ray, penetrate the strands, and terminate in typical asteroid "ribbon axons" embracing the muscle fibers. While it has been impossible to trace precisely the origin of these neurons, their courses make it highly probable that they arise, along with nerve fibers to other sets of muscles in the ray, from lateral motor centers similar to those described for *Astropecten* by J. E. Smith (1950). No fibers have been found crossing the nodule from the wall of the stomach. It may thus be concluded that the coördinated action of the retractor muscles and of the muscles in the wall of the stomach does not depend on common pathways of innervation.

The effects of x-irradiation on the pupae of the yellow mealworm, Tenebrio molitor Linn. ALAN PRIEST BROCKWAY.

In these experiments a genetically mixed culture of larvae was maintained at room temperature on a mixture of white flour and dried brewers yeast. Each morning the culture was cleared of pupae and then every four hours the pupae were collected and irradiated. Doses ranging from 500 r to 20,000 r were given at the rate of 2,500 r per minute.

All pupae used as controls or given 500 r and 1,000 r hatched normally. Of those given 2,000 r only 33% hatched normally. All pupae given 2,500 r to 20,000 r hatched abnormally. The region between 1,000 r and 2,500 r seemed to be quite critical. There was also an effect on the length of the pupal stage. The average length of pupation for the controls was 8.15 days whereas that of the pupae given 500 r was 9.0 days, 1,000 r to 4,000 r was 10.0 days and 5,000 r

to 20,000 r was 11 days. Furthermore at doses from 5,000 r to 20,000 r only 50% of the pupae hatched. Work is being carried on currently to determine the radiosensitivity of the pupae at ages other than within four hours of formation.

At 2,500 r the emerging adult was unable to shed the pupal cuticle, which remained in a mass on the tip of the abdomen. An increase of 500 r (3000 r) inhibited the shedding of the cuticle from most areas of the body. This was quite noticeable on the elytra and wings and even if the cuticle was removed with forceps these structures did not expand. The tanning process of the new cuticle was not complete in any of the adults given more than 3,000 r in the pupal stage. Only some of the cuticle was tanned giving the animal a mottled appearance and many soft areas. At times there also appeared blisters on the elytra and wings. It appeared that in the elytra the epidermal gland poured down an excess of cuticlin. The blisters on the wings were filled with a fluid which may be hemolymph, though there have been observed no hemocytes in the fluid.

Chromatographic study of crystalline style amylase.[1] ALFRED B. CHAET.

During the course of an investigation it became necessary to determine whether the amylase activity observed in the crystalline style of *Mya arenaria* was due to α-amylase, β-amylase, or a mixture of the two. The technique used in this study was that of paper chromatography followed by incubation with the enzyme's substrate. Descending type chromatograms were run in the dark at $18 \pm 1°$ C. on strips of Whatman No. 1 filter paper. After the front had descended a certain distance, the paper was air dried and placed face down in close contact with an agar-substrate film. This substrate film was prepared by allowing a 4% solution of agar (buffered at pH 7) containing 2% potato starch to gel on a large glass plate. The chromatogram-substrate system was sealed and incubated for 36 hours at 30° C. The position of α- and β-amylase was observed after removing the chromatogram and spraying (either paper or agar-substrate sheet) with a dilute solution of iodine-potassium iodide. Two satisfactory solvents were found for distinguishing between α- and β-amylase. It was shown, using a mixture of ether and phosphate buffer (pH 6.6), that α-amylase has a R_f. of 0.66, whereas β-amylase exhibits no activity. When using a 38% saturated solution of $(NH_4)_2SO_4$ as the solvent, α-amylase had a R_f. value of 0.02 and β-amylase, 0.27. Although in both solvents the spots are elongated, there is no difficulty in distinguishing the difference between the two enzymes. Experimental analysis of Mya crystalline styles shows that all of the amylolytic activity previously observed is due only to α-amylase.

Mechanism of toxic factor release.[2] ALFRED B. CHAET.

Additional experiments were carried out dealing with the characteristics of the toxic substance obtained from scalded worms (*Phascolosoma gouldii*) and an attempt made to study the mechanism of toxin release. The results indicate that in scalding experiments heat *per se* does not produce the toxin by changing a non-toxic molecule into a toxic one, for when suspensions of washed cells taken from the coelomic fluid are ruptured (by homogenization) in the absence of heat a toxin is released which, when injected into normal worms, causes death in 62 hours. This toxin has the same characteristics previously reported for the toxic factor extracted from scalded worms. It is a heat-stable, non-dialyzable molecule which precipitates in saturated $(NH_4)_2SO_4$. It appears from these and other experiments that the toxic factor is normally present in the form of a toxic molecule found within the coelomic fluid cells and that rupturing the cell by heat, crushing, or hypotonic solutions is sufficient to merely release this substance which, when allowed to circulate through the rest of the organism, results in death. When using *Limulus* as a test object it has been shown than intravenous injection of from 0.002–0.003 ml. of toxin per gram of *Limulus* is sufficient to cause death in less than 24 hours. This test object may prove useful in studying the mode of action of the toxic factor. The stability of the toxin is illustrated in experiments whereby samples stored in a frozen state for 10 months

[1] Supported by funds from the State of Maine, Dept. of Sea and Shore Fisheries.

[2] Supported in part by funds from Boston University School of Medicine and Coe Research Fund.

showed no apparent loss of biological activity. By differential centrifugation experiments it was possible to separate the coelomic fluid cells into 5 distinct fractions, but no single fraction was the storehouse for the toxic factor since at least 4 out of 5 released the toxic material when heated *in vitro*.

A combined effect of urea and borate buffer on uricase activity.[1] AURIN M. CHASE.

The effect of urea and borate buffer concentrations on the oxidation of uric acid by uricase was studied by measuring the change in extinction at 300 mμ using the Beckman DU spectrophotometer, maintained at 26° C. by water cooling. Reaction mixtures contained 0.33 mg./ml. of uricase (Worthington crude preparation) and 8 μg/ml. of uric acid, plus the desired urea concentration; all dissolved in pH 9 borate buffer of the required molarity. These molarities were 0.10, 0.23, 0.37, and 0.50; each used in conjunction with urea concentrations up to 9.0 *M*. After adding uricase the extinction was measured for 10 minutes, during which time the reaction was zero order. The slope of the resulting line was taken as the measure of enzyme activity.

Borate buffer by itself had an inhibitory effect on uricase activity, the initial rate in 0.5 *M* being about 75% that in 0.1 *M* concentration. With 4.8 *M* urea present, activity in 0.5 *M* borate was about half that in 0.1 *M*. In 0.5 *M* buffer regular, increasing inhibition occurred with increasing urea concentrations whereas, in 0.1 *M* buffer, there was no inhibition until nearly 4.0 *M* urea concentration was reached. Then activity dropped to zero as 8.0 *M* urea concentration was approached. The intermediate borate concentrations gave intermediate results.

Uricase inactivation at the lower urea concentrations was essentially instantaneous. Above 6.4 *M*, however, a slower inactivation occurred in addition to the rapid effect. The pH of all four buffers was increased from 9.0 to 9.7 by added urea up to 9.0 *M*, and this may well be a complicating factor.

It is known (Canellakis and Cohen, 1955) that intermediates and end products of this reaction may differ in different buffers. The present results show, in addition, that the kinetics observed for urea inactivation can be considerably influenced by the concentration of the buffer used.

Dimethylated dioxypurines and/or x-ray inhibition of Arbacia egg development. RALPH HOLT CHENEY.

Current dimethylxanthine and x-ray inter-relationship studies regarding their inhibitory action, separately and combined, on growth phenomena were done using the *Arbacia punctulata* egg as the test material. *M*/400 theophylline (1:3 CH_3 2:6 dioxypurine) and *M*/400 theobromine (3:7 CH_3 2:6 dioxypurine) and 30,000 r x-ray dosage were employed for comparison with reports (Cheney 1948, 1949) on inhibition by Tp, Tb, and caffeine (1:3:7 CH_3 2:6 dioxypurine); upon caffeine with x-ray (Cheney and Rugh 1954); and the report by Cheney (1955) on purine ($C_5H_4N_4$). Tb is somewhat more inhibitory than Tp at *M*/400. 30,000 r delays but does not arrest egg development prior to the stage at which *M*/400 of either drug separately stops growth without irradiation. 30,000 r does not affect the fertilizing power of the sperm. Eggs were x-rayed before fertilization.

Development of non-irradiated eggs, x-irradiated with or without drug; drug-treated 30 minutes with or without x-rays; also, immediate mixing for insemination in drug without pretreatment, was observed until controls reached pluteus. Subsequent to experimental conditions cited as prior to mixing with normal sperm, equivalent egg numbers were transferred to stender dishes, inseminated by normal sperm, and maintained in running sea water (SW). Developmental time and form were recorded in SW, SW with drug, irradiated SW without drug, and irradiated SW with drug.

The over-all effect of x-irradiation alone, *M*/400 Tb and Tp with and without 30,000 r was delay in development: first, at prophase amphiaster; second, at blastula-gastrula sequence; and third, to eventually arrest development. No evidence was found to indicate any significant synergistic, summation, nor antagonistic action between either Tb or Tp and 30,000 r irradiation although each of the three factors is separately inhibitory. This suggests that their individual effects are via different mechanisms.

[1] Supported in part by a National Science Foundation grant.

The distribution of mitochondria and lipid droplets during early cleavage in Ilyanassa obsoleta. A. C. CLEMENT AND F. E. LEHMANN.

Mitochondria were identified by their size and affinity for Janus green. In centrifuged eggs they form a band between two layers of clear cytoplasm. The Janus green stain of the mitochondrial band may be fixed with OsO_4. The normal localization of mitochondria and lipid droplets has been followed from the one-cell stage through the time of formation of the fourth quartet of micromeres. The position of mitochondria was revealed by vital staining with Janus green (1 : 100,000 solution), and that of lipid droplets by staining with Sudan III or OsO_4.

Before cleavage the mitochondria are concentrated mainly in a broad cytoplasmic cap around the animal pole. Each of the first 4 blastomeres shows abundant mitochondria. All of the cells of the first three quartets of micromeres, and their derivatives, also receive abundant mitochondria. After the formation of the first and second micromere quartets, the mitochondria in the macromeres are massed in a broad crescentic zone where the cytoplasmic cap meets the yolk zone. The mesentoblast cell 4d receives numerous mitochondria; the fourth quarter entoblasts (4a, b, c) also contain numerous mitochondria, whereas the 4A, B, C and D macromeres show relatively few.

Lipid droplets are concentrated near the animal pole of the uncleaved egg and are apportioned rather evenly to the first 4 blastomeres. In the early macromeres the lipids form a crescentic band between the cytoplasmic and yolk zones. In all of these respects the general pattern of lipid distribution is rather similar to that of the mitochondria. However, a gradient of lipid distribution appears during the formation of the micromere quartets. The first quartet micromeres receive very few lipid droplets, the second somewhat more and the third still more. The 4d cell receives nearly all of the lipids from the 3D macromere; there is a more even distribution of lipids at the subdivision of 3A–3C.

The uptake and distribution of radioactive alloxan in islet and other tissues of the toadfish. S. J. COOPERSTEIN, ARNOLD LAZAROW AND WILMA LAUFER.

As part of a broad research program on the problem of diabetes mellitus we have been interested in determining the mechanism by which alloxan selectively kills the insulin-producing cells of the islets of Langerhans and thereby produces diabetes. One of the immediate problems in elucidating the mechanism of alloxan action is to determine whether this chemical agent is selectively concentrated by the insulin-producing beta cells or whether the selectivity of alloxan for the beta cells is due to their specialization for insulin synthesis. The toadfish is ideally suited for these studies. Whereas in mammals the islet tissue is distributed throughout the pancreas and constitutes only 1% of its total weight, in the toadfish the islet tissue is segregated into a discrete mass which is separated from the pancreatic acinar tissue. Therefore we have studied the uptake of radioactive alloxan by the principal islet and other tissues of the toadfish, *Opsanus tau.*

We have developed suitable techniques for injecting alloxan directly into the circulation through a gill arch vessel. In this manner we have determined the distribution of radioactive alloxan in the various tissues as early as 90 seconds after injection and at various later times (2, 2 and ½, 5, 15 and 30 minutes). The absolute amount of radioactivity found in the various tissues increased rapidly and then gradually decreased with time. Its relative distribution among the different tissues, however, did not change significantly. Blood had the highest activity at all times. The activity in the other organs decreased in the following order: gill, heart, kidney, islet, brain, liver and skeletal muscle. The islet tissue had only ¼ to ⅓ of the radioactivity found in blood, and at no time did its radioactivity exceed that which would have resulted from uniform distribution of the radioactive alloxan throughout the body.

Phosphorylase system in the lobster.[1] ROBERT W. COWGILL.

Phosphorylase catalyzes the reversible formation of glycogen and inorganic phosphate from glucose-1-phosphate. This enzyme exists in tail muscle of the lobster in three forms: phosphorylase a is active enzymatically, phosphorylase b requires muscle adenylic acid for activity,

[1] This work was supported by a grant from the National Institutes of Health.

and phosphorylase c is completely inactive. The relative levels of these forms of the enzyme change with the physiological condition of the animal (Cowgill and Cori, 1955) but the principal form extracted from the normal lobster is phosphorylase c.

Phosphorylase c has been separated from phosphorylase a and phosphorylase b by ammonium sulfate fractionation of crude muscle extracts. It has been tested in the presence of a wide variety of metal ions and nucleotides and it has been found completely inactive. The ammonium sulfate fractions also contain enzymes for the interconversion of the various forms of phosphorylase. Two of these are activating enzymes. Activating enzyme No. 1 converts phosphorylase c to phosphorylase b; this enzyme requires Fe^{++}, Cd^{++}, Pb^{++}, or VO^{++} for activity. Activating enzyme No. 2 converts phosphorylase b to phosphorylase a, and requires Mn^{++} and adenosine triphosphate for activity. Both the metal ion and nucleotide requirement are highly specific for the latter enzyme. In addition to these enzymes, there is at least one inactivating enzyme that converts phosphorylase a to phosphorylase c. This enzyme requires Mn^{++} or certain other divalent cations for activity, but unlike the second activating enzyme it is completely inhibited by fluoride ions. All of the activating and inactivating enzymes were inhibited by ethylenediaminetetraacetic acid.

It is interesting that these enzymes that interconvert the various forms of lobster phosphorylase also are capable of interconverting phosphorylase a and phosphorylase b from rabbit muscle. That is, the inactivating enzyme of lobster will convert rabbit phosphorylase a to phosphorylase b (but not to a phosphorylase c form); and activating enzyme No. 2 of lobster will convert rabbit phosphorylase b to phosphorylase a.

The action of Nessler's reagent and ATP on extracted and denatured muscle.[1] DAVID DIBBELL AND HOWARD HOLTZER.[2]

The following experiments suggest that Nessler's reagent has more than one mode of action on the muscle model; one simulates the action of ATP, the other does not.

Muscle fibers contracted maximally in either ATP or Nessler's and inspected under phase, exhibited extreme contraction bands. Such contracted fibers may be stretched to, and maintained at, 300% of rest length by treatment in Nessler's followed by versine (Lacki). Stretched fibers can be recontracted by Nessler's but not by ATP. If a contracted fiber is stretched an expanded contraction band pattern is observed. Subsequent changes in length do not change this contraction band pattern. Fibers lightly prefixed in formalin to inhibit contraction and extended in Nessler's, yield the stretched A, I, Z and H band pattern. Thus ATP or Nessler's induced maximal contractions produce the same irreversible submicroscopic reorganization which is expressed cytologically as contraction bands.

Differences between ATP and Nessler's were demonstrated by treating muscle models with heat, formalin, acetone, absolute alcohol, low pH, and extracting in 0.5 *M* KI, 0.9 *M* LiCL or urea. In all these instances, Nessler's induced contractions after the muscle proved refractory to ATP. For example, 0.5 *M* KI extracted fibers do not contract to ATP after one hour, but react to Nessler's after 6 hours. Extension in Nessler's followed by contraction can be observed in treated fibers that will not contract from rest length. For example, fibers extracted in 0.5 *M* KI for two weeks though not contracting when initially placed in Nessler's, when stretched, contract vigorously. Nessler's will contract living muscle, whereas ATP will not. These experiments indicate that Nessler's, in addition to its effect on the ATP-sensitive actomyosin complex, may act on the muscle fiber skeleton.

Fermentation studies in nine varieties of Tetrahymena pyriformis.[3] ALFRED M. ELLIOTT AND DARRYLL E. OUTKA.

In a search for biochemical differences in *T. pyriformis*, clones representing each of the 45 mating types of the nine known varieties were tested for their capacity to ferment the following

[1] Supported by Multiple Sclerosis Society and National Foundation for Infantile Paralysis grants.

[2] American Cancer Society Scholar.

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sugars: dextrose, levulose, galactose, mannose, sucrose, maltose, and lactose. To the basic medium, containing 0.5% proteose peptone, 0.5% tryptone and phenol red as the indicator, filtered sugars were added aseptically to make up final concentrations of 0.5%. The pH was initially set at 7.2. Light inoculations (0.2 ml.) of the organisms in log growth were dispensed into the test and control media which were then incubated at 27° C. Records of color changes were made at one, two, three and 5 days; in doubtful cases the tubes were maintained for as long as 12 days.

All clones fermented dextrose, mannose, and maltose. Levulose was degraded by all except two clones in variety 1. None fermented sucrose and only four of the five clones tested in variety 9 attacked lactose. The widest variation occurred with galactose which was fermented by all clones in varieties 1, 3, 7 and 9 but was unaffected by clones from varieties 2, 4, 5, 6 and 8.

These results indicate considerable variation in fermenting capacity among the clones tested, and although varietal differences are striking, the number of clones tested is insufficient for any final generalizations. Only after a large number of clones from each variety are examined can general conclusions be drawn.

Electron microscope studies of conjugating Tetrahymena pyriformis.[1] ALFRED M. ELLIOTT AND JOHN W. TREMOR.

The sequence of events occurring during conjugation in *T. pyriformis* (strains WH6 and WH14, variety 1, mating types I and II) was observed under the electron microscope. Concentrated suspensions of washed cells in various stages of conjugation were prepared from both homogenized and sectioned material. The former proved unsatisfactory owing to destruction of nuclear elements. Sectioned material revealed certain details of conjugation not observed with the light microscope. The organisms were fixed in 1% veronal-acetate buffered formalin and treated with 2% osmic acid. They were then concentrated, embedded in methacrylate, and sectioned.

Sections through the region of contact between the two mates showed regularly spaced protoplasmic bridges of approximately 0.2 micron in diameter which could conceivably serve for the exchange of cytoplasmic materials. These were well established at the time of the third prezygotic division and possibly earlier. Since they possess about the same diameter and spacing as cilia, it is possible that they were derived from them. The morphology of the membranes in the region of nuclear exchange was clearly seen. Most of the nuclear stages occurring during conjugation were observed. Examination of early and late macronuclear anlagen stages revealed structure which could not be clearly interpreted from the preparations. Chromosomes were readily observed but showed nothing that had not already been seen under the light microscope. It is hoped that with better fixation other details may be seen that will supplement our knowledge of the morphology of this and other ciliates.

Influence of hematoporphyrin and phenol on x-radiation sensitivity of Paramecium. FRANK H. J. FIGGE[2] AND RALPH WICHTERMAN.[3]

In previous experiments, hematoporphyrin solutions containing phenol as a preservative had been employed to alter the radiation sensitivity of *Paramecium caudatum*. Paramecia placed in the solutions of hematoporphyrin prior to irradiation exhibited an LD 50 (24 hours) of 18 kr while the control groups required a dose of 340 kr. Experiments reported here were designed to ascertain the influence of phenol and hematoporphryin on radiation sensitivity when used in-

[1] This investigation was supported by research grants from the National Institutes of Health (PHS G3588C3) and the Horace H. Rackham School of Graduate Studies, University of Michigan.

[2] Supported by grants from the Anna Fuller Fund and the American Cancer Society, Maryland division.

[3] Part of a project aided by a contract between the Office of Naval Research, Department of the Navy, and Temple University (NR 135-263) and the Committee on Research, Temple University.

dependently and combined. The conditions of the radiation experiments were essentially the same as those described in our earlier publications. *Paramecium bursaria* and *P. multimicronucleatum* were irradiated in Nylon syringes so that samples could be taken at various dose intervals up to 100,000 r. The highest dose killed none of the controls. All of the animals placed in porphyrin and phenol combined were killed by 50 to 100 kr doses. The LD 50 for *P. bursaria* was 16 kr, the same as observed previously. The 1:20,000 hematoporphyrin solutions without phenol had relatively little observable effect since nearly all animals of both species survived the maximum dose given. Animals placed in 1:10,000 phenol solutions corresponding to the concentration of phenol in the porphyrin and phenol mixtures were sensitized to x-radiation. None of the animals survived a dose of 33 kr. The LD 50 for the phenol-treated group was even lower (14 kr, *P. bursaria*) than the solutions containing hematoporphyrin and phenol.

It was also observed that the irradiated animals in the phenol solution survived much longer (12 hours) when kept in Nylon syringes and deprived of air while specimens that were expressed from syringes into spot plates died within 30 minutes.

Thus the increased sensitivity of paramecia is due mainly to the phenol which is used as a preservative for the porphyrin solutions. Attempts will be made to utilize phenol in combination with porphyrin and phenol alone in cancer therapy.

The effect of argon at high pressures on the cleavage time of the sea urchin, Arbacia punctulata. CHARLOTTE HAYWOOD.

Several investigators have shown that the inert gases, nitrogen and argon, at sufficiently high atmospheric pressures can exert a narcotic effect on certain tissues and on animals, including man. A previous study of nitrogen at pressures up to 61 atmospheres upon fertilized Arbacia eggs failed to demonstrate a delay in the cleavage rate. A similar study has now been made with argon since it might be expected to be more effective than nitrogen because of its greater lipid solubility.

The method is essentially the same as that used with nitrogen at high pressures (already published). A pressure chamber which permitted microscopic observation was employed. Adequate oxygen was available in the air initially present in the chamber. Controls in air at atmospheric pressure were run simultaneously. Temperatures were 21.5° to 22.0° C.

The time required for the first cleavage to appear in 50% of the eggs was regarded as the cleavage time. In ten experiments with argon at 41 atmospheres the cleavage rates were retarded 4 to 8 minutes (average = 5.9 minutes). These values represent delays of 8 to 15 per cent (average = 11%) beyond the control cleavage times. In six experiments at 55 atmospheres of argon the delays were 9½ to 18 minutes (average = 14.2 minutes), or cleavage times 16.4 to 34% (average = 26.2%) longer than the controls. At still higher pressures the delays were greater, although pressures up to 68 atmospheres failed to give complete suppression of cleavage. Abnormalities were frequent at the pressures above 55 atmospheres.

Hydrostatic pressure *per se* is not involved with 41 and 55 atmospheres at least, as shown by earlier negative results with nitrogen and with helium at 61 atmospheres.

The enhancement of somitic muscle maturation by the embryonic spinal cord.[1] HOWARD HOLTZER,[2] JAMES LASH AND SYBIL HOLTZER.

Recent experiments in this laboratory indicated that in the chick maturation of somitic muscles was enhanced by the spinal cord but unaffected or inhibited by the notochord. This relationship prompted a re-examination of the situation in amphibian embryos, where it has been claimed that the notochord is essential to somitic myogenesis. The following experiments will demonstrate that in salamander, as well as chick embryos, the spinal cord stimulates the growth of somitic muscle, whereas the notochord is inert.

Clusters of 6 to 10 isolated somites from tail-bud embryos were implanted into the dorsal fin of young larval hosts. At time of sacrifice three to 5 weeks later, small strands of muscle and pronephric tissue were found in the quasi-culture chamber of the dorsal fin of the host.

[1] Supported by U.S.P.H. Service and Multiple Sclerosis Society grants.

[2] American Cancer Society Scholar.

Implanted clusters of somites plus notochord differentiated into a similar small mass of muscle. Irregular and inconstant mounds of cartilage adhering to the notochord and scattered kidney tubules were also present. In contrast, the same mass of somites plus a piece of spinal cord cultured in the dorsal fin formed a large mass of well differentiated muscle, similar to what the somites would have formed if left *in situ*. Cartilaginous vertebral elements, including precociously formed centra, were also present. The enhancement of muscle growth is not a generalized property of neural tissue. Implants of forebrain and somites yielded the small numbers of muscle strands found in cases of somites alone or somites plus notochord. That this action of the embryonic spinal cord on muscle growth need not be mediated by motor nerves is indicated by implants of somites from stage 33 embryos. Sizeable muscle masses developed in this series though motor nerves were not present.

The relation of the cortex to the formation of a perivitelline space in the eggs of Fundulus heteroclitus. CHARLES W. HUVER.

Unfertilized Fundulus eggs were centrifuged in an effort to determine if the cortically located cytoplasm is necessary for the formation of a perivitelline space. An International clinical centrifuge was used. Temperatures ranged from 22° C. to 26° C. during the experiments. All eggs were in a medium of 0.95 *M* sucrose solution. The control group consisted of 41 unfertilized eggs which were neither centrifuged nor pricked.

Preliminary centrifugation experiments at 846 × g. and at 3,390 × g. for 10 minutes caused the cytoplasm to concentrate at the centrifugal end of the egg. Cytoplasm could be seen because of its grey or brown color when concentrated. In all 28 eggs centrifuged in these early experiments the blastodisc formed at the concentration of cytoplasm. Cortical alveoli were densely packed in the blastodisc and a few were scattered on the egg surface. The size of the blastodisc was inversely correlated with the number of alveoli left in the egg cortex. Therefore, cortical alveoli may be regarded as indicators for the presence of cortical cytoplasm.

When 40 eggs were centrifuged at 4,320 × g. for 5 minutes, the cortex appeared to have been moved inside the egg in 15 cases. Since no cortical alveoli were seen on the egg surface, it is inferred that little or no cortical cytoplasm was present. The former cortex was clearly visible as a grey area filled with alveoli. This internally displaced cortex rounded up to form a blastodisc surrounded by yolk. Seven of the 15 eggs with internal blastodiscs were pricked with a glass needle 25 μ in diameter. In none of these eggs was there any formation of a perivitelline space. While in the controls and in the 25 other experimentals which had some cytoplasm remaining in a cortical position, a perivitelline space was formed regardless of whether they were pricked or not. These results suggest that cortically located cytoplasm is necessary for the formation of a perivitelline space.

The occurrence of a crystalline style in the marine snail, Nassarius obsoletus.[1] CHARLES E. JENNER.

The crystalline style is a specialized digestive apparatus found in all bivalve mollusks, but in gastropods its occurrence is believed to be restricted. The present report of its occurrence in *Nassarius obsoletus* is of special interest since: (1) this snail belongs to the order Stenoglossa, a group in which the style is believed not to occur; (2) this snail is frequently described as carnivorous, but the style is said not to occur in carnivorous snails; (3) this snail passes large quantities of sand and other particles of similar size through its digestive tract by peristalsis, a procedure not followed by any other snail reported to have a crystalline style.

The characterization of this snail as primarily carnivorous or as essentially a scavenger on dead animals is clearly in error for the following reasons: (1) the concentration of snails frequently encountered must require a food source far greater than provided by available animal substance; (2) the gut is frequently filled with bottom materials, mud and sand; (3) the possession of a crystalline style is primarily an adaptation for the digestion of starch, a plant product.

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

Seasonal resorption of the copulatory organ in Nassarius trivittatus and Littorina littorea.[1] CHARLES E. JENNER.

In *Nassarius obsoletus* the termination of seasonal reproductive activity is marked by the resorption of the copulatory organ in males (Jenner and Chamberlain, 1955). This has been found to be true also for *Nassarius trivittatus* and *Littorina littorea*, the phenomenon for the latter having previously been recorded for European waters (Tattersall, 1920, and others). As judged by copulatory organ resorption, *Nassarius trivittatus* from the shallow water of Eel Pond, Woods Hole, terminated seasonal reproduction by early July 1956. By the same criterion, *Littorina littorea* from mid-tide level at Nobska Point, Woods Hole, showed evidence of reproductive decline by late July; approximately 25 per cent reduction in males had occurred by August 25.

Electron microscopic observations on changes in the cortical cytoplasm after fertilization of Fundulus eggs. NORMAN E. KEMP AND MARGARET D. ALLEN.

Eggs were fixed in buffered (pH 7.4) 1% osmic acid in artificial sea water at various times: (1) before fertilization; (2) during breakdown of cortical alveoli, a process which starts at the animal pole one-two min. after insemination and is completed at the vegetative pole about one min. later; (3) after breakdown of cortical alveoli was complete; and (4) after the blastodisc was well-formed. In order to eliminate most of the yolk, eggs were fixed lightly (three-five min.) in osmic acid, then transferred to 50% sea water for removal of the chorion and bisection of the egg with iridectomy scissors. Unfixed yolk was washed out with a pipette and the partially fixed half-shells of cytoplasm and adherent yolk returned to osmic acid for about 15 min. to complete osmication, following which they were processed for methacrylate embedding and thin sectioning. Unfertilized eggs have short microvilli scattered over the surface. Some cortical alveoli are covered only by a thin layer of cytoplasm; others are embedded more deeply, some even resting against the interior yolk mass. After fertilization, alveoli come to the surface, burst and liberate their contents into the perivitelline space. Many alveoli form crater-like depressions in the surface cytoplasm during the process of extrusion. Cytoplasm may heap up to form a prominent elevated rim around an alveolar crater. Sides and floor of a crater come to be part of the surface of the egg as it smooths out after the cortical reaction is completed. The surface of the blastodisc possesses long pseudopodial processes indicative of great activity of the cortical cytoplasm. By contrast, the thin yolk gel layer peripheral to the blastodisc has a perfectly smooth surface.

Dehydrogenase activity in developmental stages of Asterias as measured with tetrazolium salts. EVELYN KIVY-ROSENBERG AND BENJAMIN W. ZWEIFACH.

A quantitative estimate of endogenous dehydrogenous activity during developmental stages of Asterias was sought using tetrazolium salts as indicators. The toxicity of the tetrazoles made such studies not feasible. As a result, an attempt was made to investigate specific substrate-dependent dehydrogenases at given stages (uninseminated ova, inseminated ova, blastulae, gastrulae). A method similar to the frozen tissue-slice technique was employed in which most endogenous activity is eliminated by freezing, and circumvents the problem of toxicity.

Having been removed from deep freeze, samplings of chosen developmental stages were thawed at room temperature. These were incubated anaerobically at 37–38° C. in a medium containing sea water, DPN, nicotinamide and one of a series of substrates (which had been used previously in tissue work): succinate, alpha-glycerophosphate, glucose, glutamate, malate, lactate, beta-hydroxybutyrate. No cofactors were used with succinate. Two tetrazolium salts were employed: 2, 3, 5 triphenyltetrazolium chloride (TTC) and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Final concentration of tetrazolium in the medium was under 0.5%. The amount of formazan was determined colorimetrically by a spectro-

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

photometer. The substrate-dependent dehydrogenase activity was expressed in terms of micrograms per cubic milliliter of ova (or embryos).

It is evident that particular substrate-dependent dehydrogenases are more metabolically active than others and that there is a quantitative variation during development. Although the absolute quantity of reduced tetrazolium varied somewhat from one batch of eggs (or embryos) to another, it appears when TTC was used as the acceptor, that malate dehydrogenase activity is highest, with beta-hydroxybutyrate and alpha-glycerophosphate next in order though definitely lower. Other substrates yielded negligible values or none at all. With INT, malate, alpha-glycerophosphate and beta-hydroxybutyrate were highly active. Other substrates gave positive reactions but of much lower intensity. Limited observations indicate that alpha-glycerophosphate, malate, and beta-hydroxybutyrate-dependent dehydrogenases become less active soon after insemination and then increase again at gastrulation.

The chemical nature of bound oxygen in hemerythrin and in hemocyanin.[1] IRVING M. KLOTZ AND THEMIS A. KLOTZ.

Since ferrous ion can be released from deoxygenated hemerythrin and ferric ion from oxyhemerythrin, it has been suggested that the bound oxygen in the oxygenated protein is in the form of peroxide ion. Several tests for peroxide ion have been carried out, therefore. The most delicate test for H_2O_2 is the formation of a yellow color with a solution of TiO_2 in dilute sulfuric acid. To five drops of Ti(IV) test reagent in a spot plate was added, therefore, some oxyhemerythrin crystals. An orange color formed immediately around the protein; on further mixing the color was diluted to a strong yellow. Corroboration of the presence of peroxide ion was also obtained with the benzidine test. A few drops of a 4% solution of benzidine in glacial acetic acid were placed in a spot plate. To this solution were added a few milligrams of horse-radish peroxidase and then some crystals of oxyhemerythrin. A blue color developed immediately in the vicinity of the hemerythrin. Concentrated solutions of crystalline oxyhemerythrin, as well as laked blood, also gave positive benzidine tests. In contrast deoxygenated hemerythrin did not give the blue color. Both tests thus show that peroxide ion is released from oxyhemerythrin in acidified solutions.

In hemocyanin valence changes of the copper on oxygenation suggest that the bound oxygen is in the form of perhydroxyl free-radical ion. This species is a reactive intermediate known to appear in the metal-catalyzed decomposition of peroxide. Oxyhemocyanin was added, therefore, without peroxidase, to a solution of benzidine in glacial acetic acid. At 0° C., a stable blue color, characteristic of oxidized benzidine, was obtained, as would be expected if perhydroxyl radical were released from hemocyanin.

Crystallization of Busycon hemocyanin.[1] IRVING M. KLOTZ, THEMIS A. KLOTZ AND GEORG H. CZERLINSKI.

Attempts to prepare crystalline hemocyanin from the blood of *Busycon canaliculatum* by standard procedures used for other species have not proved successful. It has been found now, however, that an adaptation of the heparin method described by S. Cohen (1942) for other macromolecules does produce crystals.

Blood drained from the tissues of the conch is filtered through glass wool and then centrifuged at 4° C. at 2000 r.p.m. in the International Refrigerated Centrifuge. The solution is then decanted and dialyzed against distilled water at 4° C. for two days, with frequent replacement of the water outside the dialysis bags. The hemocyanin is then separated from the blood by ultracentrifugation at 35,000 r.p.m. in the Spinco Model L. The colorless supernatant is discarded, the concentrated hemocyanin at the bottom of the tube is redissolved in distilled water and the ultracentrifugation repeated. After the third ultracentrifugation, the

[1] This investigation was supported in part by a research grant, RG-4134, from the National Institutes of Health, Public Health Service.

concentrated hemocyanin is removed and to it is added solid sodium heparin, or a 10% aqueous solution, to a final concentration of 3%. Turbidity appears immediately but the mixture is permitted to stand overnight. The solid is then separated by centrifugation at 1500 r.p.m. Examination under the microscope reveals crystals, perfect ones being in the shape of hexagons.

Even this crystalline hemocyanin, in aqueous solution, shows two widely separated components in the Spinco analytical ultracentrifuge Model E. It is likely that the faster moving one is a dimer or higher aggregate of the monomeric form.

Pathways of glucose-C^{14} utilization in eggs of Arbacia, Mactra, and Chaetopterus. M. E. KRAHL, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

From experiments with glucose-1-C^{14} (G1), glucose-2-C^{14} (G2), and glucose-6-C^{14} (G6), the authors reported here (1955) that: (a) glucose is oxidized in Arbacia eggs principally via the TPN shunt, the ratio of $C^{14}O_2$ from G6 to that from G1 being 0.07 for unfertilized and 0.12 for fertilized eggs; the glycolytic pathway is more important in 24 hour-old swimming embryos where the ratio is 0.28; (b) C^{14} from glucose appears in nucleoprotein; (c) dinitrocresol (DNC) inhibits glucose oxidation by the shunt, tending to divert glucose-6-phosphate into the glycolytic pathway; DNC also inhibits glucose-C^{14} conversion to nucleoprotein. These findings have been extended for Arbacia and found to apply in general to eggs and embryos of Mactra and to embryos of Chaetopterus. Representative total c.p.m. collected after 2 hr. incubation of 80 mg. eggs at 20° in 4 ml. sea water containing glucose-C^{14} (0.0006 *M*; 600,000 c.p.m.) are as follows. For Arbacia, just fertilized, in DNA from G1, 200, from G6, 700; in RNA from G1, 200, from G6, 400. For 24 hr. embryos, in DNA, from G1, 20,000, from G6, 18,000; in RNA, from G1, 12,000, from G6, 12,000. For Mactra the total c.p.m. into respiratory CO_2 with 0 and 0.0001 *M* DNC present were, respectively: unfertilized, from G1, 200, and 300; G2, 100 and 500; G6, 25 and 200; just fertilized, G1, 300 and 400; G2, 200 and 1600; G6, 200 and 700; 24 hr. embryos, G1, 16,000 and 22,000; G2, 7000 and 13,000; G6, 4000 and 11,000. The total c.p.m. into nucleoprotein of 24 hr. embryos with 0 and 0.0001 *M* DNC were, respectively: from G1, 27,000 and 4900; G2, 33,000 and 5900, G6, 33,000 and 6600. For Chaetopterus the total c.p.m. into respiratory CO_2 with 0 and 0.0001 *M* DNC were, respectively: for 24 hr. embryos, from G1, 5500 and 5000; G2, 1500 and 6500; G6, 800 and 3600. The total c.p.m. into nucleoprotein of 24 hr. embryos with 0 and 0.0001 *M* DNC were, respectively: from G1, 13,000 and 1400; G2, 16,000 and 2100; G6, 18,000 and 2000.

Improved fixing and staining methods for cellular structures in Ilyanassa eggs. F. E. LEHMANN.

1. *Fixation.* Previous studies had shown that in Amoeba fibrous structures in cytoplasm are not sufficiently preserved by osmic or strongly acidic fixatives. A suitable rapidly penetrating and partly dehydrating fixative was found in a mixture of acetone and formalin; good preservation of cytoplasmic fibers was shown by the electron microscope. For Ilyanassa two mixtures were tried. A mixture of 80 ml. of 20% formaldehyde and 16 ml. of acetone is sufficient to fix fibrillar structures in the cytoplasm (cytoplasmic reticulum and asters). A slightly acidified fixative (80 ml. of 20% formaldehyde, 16 ml. of acetone, and 0.5 ml. of glacial acetic acid) gave good preservation also of interphase nuclei, spindles with chromosomes, and asters. In contrast to this, OsO_4 fixation of Ilyanassa eggs destroys astral structures, which are visible in living eggs. In order to stabilize structures containing nucleic acids and to prevent shrinkage by increasing rigidity of cell parts, chromic acid was added to the fixative (2 ml. of 10% chromic acid for every 5 ml. of fixative) 20 minutes after eggs had been placed into the fixative. After 5–20 minutes of exposure to this combination, eggs were rinsed for 1–2 minutes in distilled water; they were then ready for staining and mounting.

2. *Staining* by bromphenol blue-sublimate (after Mazia, Brewer and Alfert) showed cell boundaries, cytoplasmic reticulum, spindles and astral fibers clearly. For staining of total mounts of Ilyanassa eggs the original mixture (0.05 mg. bromphenol blue, 5 g. sublimate and 50 ml. H_2O) was diluted 20 times. Eggs were stained 10–20 minutes, transferred to 0.5% acetic acid for 20 minutes, and then to tap water for 5–20 minutes. They were then passed through alcohol, cleared in xylene and mounted in Canada balsam. Besides the blue cytoplasmic

structures the yolk granules stained partly purple, violet and green. The shape of the egg is very little distorted as compared with the living stages.

Factors inhibiting metamorphosis in tadpoles of the tunicate Amaroecium constellatum. WILLIAM F. LYNCH.

Tadpoles were placed in sea water solutions of 0.001 *M* potassium cyanide, 0.001 *M* sodium azide, 1.5% urethane (all at a pH of 7.8–8.0) and in sea water acidified to pH values of 4.8, 5.2 and 5.7. The number undergoing metamorphosis was determined: (1) after the last control had begun metamorphosis (30–70 min.) in eight observations, (2) at 7 hours, and (3) at 21 hrs. in three experiments. (1) After the last larva in the controls had begun metamorphosis the inhibition was: urethane, 100%; azide, 98%; cyanide, 97%; acidified sea water (pH = 4.8–5.2), 94%. Inhibition was completely reversible on removal of the tadpoles to sea water. (2) By 7 hours none of the tadpoles in urethane or azide (other experiments) and only 2.8% of those in cyanide and 8.3% of those in acidified sea water (pH = 5.7) had begun metamorphosis. Inhibition was reversible. (3) In another set of experiments inhibition was 100% in urethane and in acidified sea water (pH = 5.2) at 21 hrs. Larvae removed from either of these solutions showed a tendency for persistent swimming. Those removed from urethane changed their axes from 45 to 90° by 24 hrs. Those removed from acidified sea water only partially metamorphosed and then died. Inhibition was incomplete at 21 hrs. in azide and in cyanide. Unmetamorphosed tadpoles removed at 21 hrs. from azide developed normally and those from cyanide at a somewhat retarded rate.

Tadpoles placed in sea water solutions of 0.01% 2,4-dinitrophenol and 1.5% thiourea (both at a pH of 7.8–8.0) began metamorphosis and tail resorption as quickly as the controls, but subsequent stages were inhibited. The larvae contracted, became ovoid and the tunic imbibed much sea water; none of them changed their axes nor elongated by 24 hrs. Inhibition was completely reversible when tadpoles were removed from dinitrophenol at six hrs. or from thiourea at 1.5 hrs. Tadpoles in 0.15% chloral hydrate (pH = 8.0) showed 17% inhibition when the last larva of the controls had begun metamorphosis but they changed their axes from 10 to 90° by 24 hours; subsequent development was somewhat retarded.

A North American record of Rhopalura sp. (Orthonectida: Mesozoa), a parasite of the nemertean Amphiporus ochraceus (Verrill). NORMAN A. MEINKOTH.

On July 3, 1956, a specimen of the armed nemertean *Amphiporus ochraceus* (Verrill), taken from among growths of the compound ascidian *Amaroucium pellucidum* dredged off Nobska Point near Woods Hole, Mass., was found harboring a strange parasite. These minute, ciliated, cylindrical, annulated organisms were noted both within the body of the host and swimming beside it on the slide.

The parasite measured 126 μ by 18 μ. Its body consisted of an outer layer surrounding an inner core of large cells, 19 to 23 in number, disposed in a single row anteriorly and double posteriorly, extending the length of the organism. Constrictions of the outer layer divided the body into a series of annuli, all of which with certain exceptions contained numerous refringent granules. Three annuli constituting an anterior cone were followed by two prominent annuli, each delimited by deep furrows. Behind these followed a series of six large annuli, each succeeded by a smaller granule-free annulus. The remaining posterior part was rounded, and somewhat longer than wide. All annuli bore cilia. Those on the first annulus were held rather rigidly anteriad as a tuft, while those on the posterior part, longer than the other body cilia, did little vibrating and trailed behind. The remainder of the body cilia, of about equal length and distribution, beat actively. All individuals encountered were essentially identical in the above characteristics. To date, of 171 more *A. ochraceus* examined, none has been found harboring the parasite.

Consultation with the literature indicates that these animals are females of a species of the genus *Rhopalura,* order Orthonectida, phylum Mesozoa. The species is similar to if not identical with *R. metchnikovi* Caullery and Mesnil 1901 or *R. linei* (Giard) 1879. This is believed to be the first reported occurrence of an orthonectid from North America.

Studies on accelerator and retarding factors of one species on the developing ova of an unrelated form.[1] VALY MENKIN, GABRIEL MENKIN AND LOUISE MENKIN.

Recent observations have indicated the presence of two distinct factors in the aqueous homogenate of the ovaries of *Arbacia punctulata*. These two factors conceivably play a significant rôle in the regulation of cell division. One of them markedly accelerates the rate of cleavage of sea urchin ova; the other displays considerable retarding activity of cleavage development. Since from a single homogenate two opposed factors are obtained by methods previously described, it is conceivable that the rate of cleavage of the fertilized ova is a resultant of these two opposed effects.

In a new series, it has now been shown in 10 distinct experiments that the accelerator factor derived from the homogenate of sea urchin ovaries speeds up the cleavage of the ova of a mollusk, *Spisula solidissima*. The accelerator factor is obtained by centrifuging the aqueous homogenate at about 10,000 r.p.m. in a Servall angle centrifuge for about one hour. The supernatant is then dialyzed against distilled water in a refrigerator for several days. The diffusate contains the accelerating factor. Its activity on the ova of *Spisula solidissima* has yielded the following results: the average number of ova is about 150 per cent greater in the experimental than that of the controls in the two-blastomeric stage. In the succeeding segmentation the incidence averages 100 per cent in the experimental over that of the controls. In a series of 13 experiments with the retarding factor of Arbacia ovaries on Spisula ova, the average retarding effect on the two-cell stage is 60.9% and 38.7% in the succeeding cleavage stage. Thus the factors obtained from an echinoderm are equally effective on the developing eggs of a mollusk, indicating that basic substances are evidently involved in the mechanism.

Further studies on some factors concerned in the regulation of cell division.[1] VALY MENKIN, GABRIEL MENKIN AND WILLIAM ROGERS.

Since there is a definite suggestion that the accelerator factor seems to be a nucleotide, some studies were undertaken to determine the effect of a few derivatives of nucleic acid on the cleavage rate of Arbacia ova. The pyrimidine base uracil appears in preliminary experiments to cause an initial acceleration in the incidence of the first segmentation, the figures being 63% in the experimental and 26% in the controls. In the succeeding cleavage the experimentals reveal an incidence of 75% as against 58% in the controls. This base is to be studied further. Cytosine seems to be incapable of altering the cleavage rate. For the pyrimidine bases doses of 75 γ per ml. were employed. Adenosine triphosphate (ATP), 75 γ per ml. on *Arbacia punctulata* ova yielded, in a series of six experiments, the following results: either a retardation or an acceleration in the cleavage rate. In the first segmentation ATP induced an average retardation of 13.1%, whereas in the succeeding cleavages an accelerating effect was noted amounting to 84.4%. The effects with ATP are therefore of a different order than those obtained with the accelerator cleavage factor fractionated from sea urchin ovaries.

The effects of the accelerator and the retarding factors derived from Arbacia ovaries, on the sperms of the clam, *Spisula solidissima*, were studied. Sperms were exposed for varying intervals (33 minutes to 4 hours) to the two factors, and to sea water as control. Ova of Spisula were then added, and the incidence of cleavage subsequently determined. The results of 9 experiments indicated 44.5% cleavage in the controls; 34.7% in the dish containing the accelerator cleavage factor; and 9.8% in the one with the retarding factor. Prolonged exposure to the accelerator factor seems to injure the sperms. This detrimental effect is very pronounced with the retarding cleavage factor. However, addition of the retarding factor following fertilization induced likewise retarding cleavage effects, indicating also a probable effect on the eggs themselves.

[1] Aided by grants from the U. S. Public Health Service, Sigma Xi, and Dr. A. Wander, S.A., Berne, Switzerland.

Effect of electric current on the contraction of the chloroplasts of Spirogyra. W. J. V. OSTERHOUT.

Experiments were made on *Spirogyra* cells which contained single spiral chloroplasts. These cells were placed in 0.001 *M* NaCl solution which brought about no visible change in the chloroplasts. When a small amount of direct or alternating current (less than 0.2 milliampere) was passed through the solution each spiral chloroplast in less than ten minutes became detached, straightened, and contracted until it formed a small rounded mass. This contraction continued even after the current was turned off. A cell whose chloroplast was not visibly affected by the current sometimes showed signs of contraction after the current was turned off. This was not reversible. If the direct current was reversed there was no additional effect.

In order to make certain that these effects were not due to the heat produced in the circuit the following was done. A drop of gelatin whose melting point was 35° C. was put in the path of the current along with *Spirogyra* cells. Since the gelatin did not melt the temperature was less than 35° C. If a drop of gelatin was placed in a stender dish containing some water at 35° C. it melted. Cells of *Spirogyra* exposed to a temperature at 40° C. for one half hour in a stender dish of water showed no signs of contraction.

When the normal cells of *Spirogyra* were centrifuged the spiral chloroplasts became detached, straightened, and contracted. If the cells were centrifuged until only slight shortening of the chloroplasts was evident and removed to a stender dish of water no further contraction occurred.

Retinal action potentials in the eye of the scallop. FLOYD RATLIFF.

The scallop, *Pecten irradians*, has approximately one hundred eyes, each of which contains two retinae. Hartline found that nerve fibers from the proximal retina of an excised eye respond to the onset and continuance of illumination, while those from the distal retina respond only to the cessation of illumination. In the present study retinal action potentials were measured by placing one electrode on the posterior pole of an eye and inserting another into the eye near the margin of the lens. Upon stimulation by light the anterior portion of the eye becomes negative with respect to the posterior pole. This retinal action potential passes quickly through a maximum (approximately one millivolt when the eye is fully dark-adapted) and then subsides toward the resting level. Upon cessation of illumination the potential drops more rapidly toward the resting level. An "off" component, such as that found in many vertebrate and invertebrate eyes which respond to the cessation of illumination, is lacking. The absence of an "off" component is not due to inactivity of the distal retina, however. Simultaneous observations of retinal potentials in the eye and action potentials in the optic nerve showed that, at intensities of illumination great enough to produce a measurable retinal response, there was always a vigorous "off" discharge in the optic nerve when the light was turned off.

The course of the recovery of maximal sensitivity of the eye in the dark, following prolonged exposure to light, was determined by measuring the amplitudes of retinal potentials produced by stimuli of fixed intensity and one second duration spaced five minutes apart. Recovery is complete within about forty minutes.

Strain differences in viability following conjugation within variety 9 of Tetrahymena pyriformis.[1] CHARLES RAY, JR. AND ALFRED M. ELLIOTT.

Per cent of viable clones following conjugation of strains of variety 9 of *T. pyriformis* was studied as part of a program concerning strain relationships and genetics of biochemical differences between strains. Strains of variety 9 are known only from collections made in Panama or Colombia. Seven strains (TC. 105, 147, 156, 160, 258, 267 and 84) representing all collection sites and all five mating types were mixed in all possible combinations. Tests for selfing and for intervarietal mating were negative. Two hundred-twenty pairs were isolated singly into proteose-peptone from conjugating mixtures. Length of refractory period, amount of pairing,

[1] This investigation was supported in part by research grants from the National Science Foundation and from the National Institutes of Health (PHS G3588).

per cent of pairs giving viable clones, and stage at which death occurred for inviable pairs, were recorded.

All combinations of opposite mating types showed pairing. The refractory period of most combinations was about thirty-six hours (25° C.), although one paired at 24 hours and three at 60 hours. Per cent of pairing in various mixtures was poor to good. No relation was observed between length of refractory period, amount of pairing, and per cent of exconjugant clones. Of all crosses made, about 70% of the pairs died before fission of exconjugants, about 20% died after several fissions, and about 10% gave viable clones. Yield of viable exconjugant clones varied for different strains: *e.g.*, crosses involving TC267 gave 2% viable exconjugants; those with TC160 gave 20%. Three mating type IV strains gave 20, 13 and 2 per cent viable exconjugants. TC156 (III) and TC160 (IV) were isolated from the same original collection; they produced from all crosses 5% and 20% viable exconjugants, respectively, but when crossed with each other they gave no viable exconjugants.

Observation of cytological events associated with this widespread lethality and behavior of viable clones with continued cross and inbreeding is in progress.

Carbohydrates metabolized by cestode parasites of dogfish.[1] CLARK P. READ.

Calliobothrium verticillatum (Tetraphyllidea) and *Lacistorhynchus tenuis* (Trypanorhyncha) were removed from naturally-infected *Mustelus canis*. The worms were washed for 2 hours in several changes of filtered 40% sea water at room temperature (20–22° C.). Groups of ten worms (70 to 90 mg. of wet tissue) were transferred to Warburg flasks containing 40% sea water-bicarbonate (pH 7.2) and equilibrated for 15 minutes under 95% N_2–5% CO_2 in the 20° C. bath. Flasks were incubated for 60 minutes to determine the endogenous rate of acid production. Substrates were than added to make a final concentration of 0.01 *M* and the flasks incubated for an additional 60 minutes. Throughout each experiment readings were made at 20-minute intervals. Addition of acid to initial control flasks and to the experimental flasks showed that anaerobic metabolic gas is not produced by either of these cestode species. As indicated by an increase in acid production glucose and galactose are utilized by both tapeworms. Fructose, mannose, xylose, maltose, trehalose, sucrose, lactose, and raffinose are not metabolized. Non-utilization of the latter substrates was confirmed by analyses of the media before and after a three-hour incubation, using Roe's anthrone procedure for the sugar determinations. The rate of utilization of glucose and galactose is independent of the concentration in the range 0.001 to 0.02 *M*.

The extremely limited spectrum of carbohydrates metabolized by *Calliobothrium* and *Lacistorhynchus* resembles that of the cyclophyllidean cestodes, *Hymenolepis diminuta, Oochoristica symmetrica, Raillietina cesticillus,* and *Moniezia expansa.* Additional experiments are in progress to determine whether the dogfish cestodes resemble the cyclophyllideans in requiring the inclusion of carbohydrate in the host diet for normal growth and reproduction.

Recovery from x-irradiation effects at the cellular level. ROBERTS RUGH AND JOAN WOLFF.

It is more difficult to conceive of repair of structural damage consequent to x-irradiation than to a re-synthesis by the living system of molecules rendered unusable. Recovery of function at the cellular level is conceivable to a degree inversely related to time.

Henshaw originally described the recovery of Arbacia gametes following x-irradiation. The present experiments extend his work to determine the degree of recovery following different levels of exposure and different time intervals between x-irradiation and the fertilization of the Arbacia egg.

Eggs of Arbacia were washed and concentrated into a 40-cc. volume in filtered sea water, placed in a covered plastic dish, and x-irradiated at 2160 r/min. to from 36,000 r to 86,000 r. At intervals up to three hours after irradiation eggs were fertilized and the degree of cleavage determined at 1.5 and 2.5 hours after fertilization. To do this, eggs were fixed in 10% formalin with a trace of acetic acid, in sea water. Counts were made of 200 eggs from each sample.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Johns Hopkins University, NR 119–353.

Cleavage was delayed in all eggs x-irradiated, the greater the exposure the greater the delay. However, eggs which were irradiated but not fertilized for one, two or three hours showed increasing recovery value (cleavage percentage) with increasing time. At the higher levels of exposure, which allowed no eggs to cleave, some eggs developed when the time between irradiation and fertilization was extended.

The above facts indicate that the type of damage inflicted upon the single-celled Arbacia egg by x-irradiation is reparable to a degree.

The effect of substrate on the length of planktonic existence in Nassarius obsoleta. RUDOLF S. SCHELTEMA.[1]

Veliger larvae of the mud snail *Nassarius obsoleta* (Say) were successfully reared through metamorphosis. In culture the larvae grew rapidly in 15-liter crocks with *Nitzschia closterium* (200,000/ml.) as a source of food. The length of pelagic existence extended from 20 to approximately 30 days. Planktonic life ended with the loss of the velum. Contrary to the accounts generally given for gastropod larvae, the velum of *Nassarius obsoleta* was cast off rather than resorbed. Shell height of the larvae at the time of setting varied between 0.5 and 1.1 mm.; the average was between 0.7 to 0.8 mm. Within a few days after metamorphosis, the shell darkened and become opaque. In a series of experiments, 40 cultures, each containing 20 Nassarius veligers, were maintained through metamorphosis in the laboratory. A substrate of sand and organic material ($< 140\,\mu$, sieved from natural bottom sediment of Barnstable Harbor) was added to one-half of the cultures. To the remainder of the cultures no substrate was added. In cultures of 20-day old larvae, 17% of the organisms with a substrate metamorphosed within 24 hours, while in those without substrate only 3% set during this period. Similarly in cultures containing 31-day old larvae, 90% of the larvae with a natural substrate metamorphosed, while only 19% of the larvae without substrate completed metamorphosis. Considering the average of all cultures between 20 and 31 days, in those with substrate added, 76% of the veligers set within 24 hours while in cultures without substrate only 24% completed metamorphosis within this time. These laboratory observations have considerable ecological significance since they may help to explain the distribution of newly metamorphosed Nassarius in the natural environment.

Electrophoretic separation of chromatophorotropic principles of the fiddler crab, Uca. G. C. STEPHENS, F. FRIEDL AND B. GUTTMAN.

The following observations were carried out in order to attempt to isolate and characterize the chromatophorotropic principles present in the sinus gland of the fiddler crab, *Uca pugilator*. Sinus glands were dissected and isolated in sea water as rapidly as possible until twelve glands were obtained. These were then placed on a strip of filter paper one-half inch wide and eighteen inches long which had been moistened with $M/15$ phosphate buffer at pH 7.0. The glands were then crushed and exposed to 450 volts DC at 5 to 8 ma. for 12 to 18 hours. On a separate strip in the same apparatus a sample of serum albumen was run to serve as a marker.

The results of this procedure were observed as follows. The strip of filter paper on which the glands had been placed was divided into ten equal units, five on each side of the point at which the glands had been crushed. Each unit was extracted with 0.5 cc. of sea water and 0.05 cc. of the extract was injected into each of five male fiddler crabs whose eyestalks had been removed at least twelve hours before use. The stages of the black, white, red, and yellow chromatophores of these assay animals were estimated before injection and observed again 20, 40, 60, and 120 minutes after injection of the test extract. A quite comparable assay procedure was used to study chromatophore concentrating activity of regions of the strip. In this case, the chromatophores (black, red, and yellow) of the assay animals to be used were dispersed by injection of a sinus gland extract one to two hours before injection of the filter paper extract.

By this procedure we were able to distinguish three distinct peaks of black dispersing activity, one dubious area of black concentrating activity, and at least one peak of dispersing ac-

[1] This work was done with the assistance of a summer fellowship from the Woods Hole Oceanographic Institution.

tivity and one peak of concentrating activity for the red and yellow chromatophores. At least two peaks of white concentrating activity were discernible. Each of these peaks mentioned can be stated to be distinct from any of the others by comparing the variation obtained in our several observations.

The rate of disappearance of the melanophore-dispersing hormone from the blood of the fiddler crab, Uca. G. C. STEPHENS, A. STRICKHOLM AND F. FRIEDL.

The rate of disappearance of the melanophore hormone in *Uca pugilator* was studied by the following technique. Assay animals were prepared by removing the eyestalks of male fiddler crabs at least twelve hours before they were to be used. Within this time the melanophores had become punctate so that the dispersing activity of an extract could be readily ascertained by observing the response induced on injection of the material concerned. The melanophore-dispersing hormone to be assayed was prepared by dissecting sinus glands from normal donor animals and grinding them to prepare a sea water extract.

An initial group of twenty-five to thirty animals was injected with 0.05 cc. of sea water containing the extract from one-half a sinus gland. One group of five of these animals was followed to ascertain the effect of this full strength injection. The remaining animals were divided into groups of five each. At suitable intervals after the initial injection (5 minutes, 15 minutes, 30 minutes, 60 minutes) 0.05 cc. of blood was withdrawn from each member of a group and injected into another group of five assay animals. The dispersing effect of the blood could then be followed in these secondary assay animals. As a control, blood from uninjected assay animals was withdrawn and injected into a second group of assay animals; no dispersion was obtained.

These experiments indicated that the dispersing hormone was present in the circulating blood of destalked assay animals in discernible amounts for approximately three hours after injection. In order to get a quantitative estimate of the amount present, readings of each assay group were made at fifteen-minute intervals after injection. The first seven estimates of melanophore dispersion were summed to give a measure of the effect of the extract or the blood which the animals had received. This information has been supplemented by a dilution curve measuring the effect of various concentrations of sinus gland extract, and by a measurement of typical blood volumes of the crabs (average 26.4% of body weight). This additional information permits calculation of the circulating hormone in sinus gland units.

Studies on activation in eggs of Urechis caupo, Nereis limbata and Asterias forbesi. HOWARD M. TEMIN.[1]

Activation in eggs of *Urechis caupo, Nereis limbata* and *Asterias forbesi* involves a series of linked depolymerizations of preformed layers or membranes: breaking of secondary valence and salt bonds in the cortex and disulfide bonds in the germinal vesicle. The process was studied by use of various reagents of known chemical action.

In eggs of Urechis the vitelline-fertilization membrane is an outer protein layer, soluble in non-electrolytes, and an inner calcium-proteinate layer. Activation involves breaking of secondary valence bonds in the egg cortex with a decrease in surface area; breaking of secondary electrostatic and then salt bonds in the sub-vitelline membrane layer causing membrane elevation and release of a compound which breaks disulfide bonds in the germinal vesicle.

In eggs of Nereis the vitelline-fertilization membrane is an outer layer soluble in alkaline thioglycolate and an inner layer soluble in citrate. The un-ionized jelly is secondarily bonded to the cortical gel. In solutions containing only monovalent ions (NaCl) or of agents which disrupt the cortex (sea water, pH 2.5; urea; sodium lauryl sulfate) the jelly is set free and the egg loses its depression. At a pH less than 9 the jelly passes through the outer membrane and then releases protons. At a pH of 10.5 the jelly does not pass through the membrane (Costello). Precipitation of the jelly to give a viscous mass depends on complexing with divalent ions. The germinal vesicle is soluble in alkaline thioglycolate, but not in urea, citrate, alkaline NaCl or solutions of ions.

[1] National Science Foundation Pre-doctoral Fellow.

In eggs of Asterias the easily separable vitelline membrane is soluble in alkaline thioglycolate. On activation granules are released by breaking first secondary valence and then hydrogen bonds. These granules in the presence of ions precipitate on the vitelline membrane to give the fertilization membrane, which is also soluble in alkaline thioglycolate. The fertilization membrane expands in a divalent ion-free medium.

Spermatozoa in the oviducal gland of the smooth dogfish, Mustelus canis. LOIS E. TEWINKEL.

Fertilization of the elasmobranch egg, known to be internal, was thought to occur in the oviduct anterior to the oviducal gland until Metten reported (1939) that in *Scyliorhinus canicula* the oviducal gland, itself, serves as a seminal receptacle. Metten demonstrates the presence of spermatozoa exclusively within the shell-secreting tubules of the gland and considers that sperm are swept out, together with shell material, as the egg passes through this region.

Oviducal glands of *Mustelus canis* also contain sperm. Six mature females collected between June 28 and July 11 fall into the following three groups: a) two were post-partum and pre-ovulatory; b) one had ovulated two eggs; c) three had recently completed ovulation. Living sperm were found in washings of the oviducal gland in all specimens, but were not present in washings of more anterior or more posterior portions of the oviduct. Longitudinal sections of glands from each of the three groups of females show spermatozoa singly, in small groups, or in dense clusters, in the mouths or deep within the lumina of tubules, not only of the shell-secreting type, but also in more caudal "mucous" tubules. Occasional clusters or single sperm lie near the lamellar lining of the oviducal gland, but no sperm have been seen in albumen-secreting tubules or in more anterior mucous tubules.

Unlike the oviparous *Scyliorhinus*, which breeds throughout the year, the viviparous *Mustelus* presumably mates only between the birth of pups (late April to early June) and the onset of ovulation (June to early July). One would expect, therefore, to find few or no sperm in oviducal glands after gestation is advanced. In the glands studied, even after ovulation has ceased, sperm in large numbers are present, especially in the caudal "mucous" tubules, but whether this is the case in later pregnancy cannot be answered until glands from such females are examined.

Acetylcholine and frog brain oxygen consumption. ELBERT TOKAY.[1]

Since there seems relatively little known about the influence of acetylcholine (ACh) on brain metabolism, a study of the effect of the drug on frog brain oxygen consumption (standard Warburg technique) was undertaken for the purpose of correlating the findings with electroencephalographic results.

Optic lobes and cerebral hemispheres were separated (razor blade slicing) from freshly isolated frog (*Rana pipiens*) brains. Each Warburg flask contained four optic lobes or cerebral hemispheres (from different brains) in Ringer's. Flasks were gassed with pure oxygen and equilibrated at 30° ± 0.5° C. At 10-minute intervals, readings of "normal" oxygen consumption were taken for one hour and then for two additional hours after tipping in side-arm contents (acetylcholine chloride Merck or Ringer solutions). Each ACh flask was duplicated by a Ringer flask, both containing similar parts of the same brains. Oxygen consumption ($mm.^3$ per g. of wet tissue weight) was calculated and the Q_{O_2} ($mm.^3$ O_2 consumed per g. per hour) derived from the slope of the plotted curve.

The "normal" Q_{O_2}'s are in agreement with those of previous workers. ACh was used in concentrations ranging from 10^{-2} to 10^{-6} g. per ml., in steps of tenfold dilution. Current results indicate that high concentrations of ACh depress oxygen consumption, 10^{-3} markedly and 10^{-4} somewhat less markedly. Lower concentrations (10^{-5} and below) tend to increase oxygen consumption more often than decrease it. More definitive conclusions and any differential effects on parts of the brain must await further experimentation and statistical evaluation.

[1] This investigation was supported in part by research grant B-918 from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.

A comparison of two inhibiting agents in Tubularia. KENYON S. TWEEDELL.

Regeneration in Tubularia can be inhibited with either a culture medium filtrate or with adult tissue extracts. Suspected differences in the active components were investigated.

An effective concentration of inhibitor water is dependent upon the tissue source and the concentration. Pieces of stems only showed no inhibition while amputated hydranths gave initial inhibition at a concentration of 125. Stems plus intact hydranths were the best source; the minimum effective number necessary is about 200 to 250 per 200 ml. collected for 18 to 24 hours. During this time an average drop in pH of the collecting medium of 0.5 was noted. The inhibitor strength is reduced when it is collected in boiled sea water. Refrigeration for 24 hours at 7° C. or boiling will destroy it. Adsorption with Norite A or synthetic resins will completely remove its activity but centrifugation at 21,000 G has no effect. The fresh filtrate gives a positive test with ninhydrin and the Feulgen-Schiff reagents. The regenerating stems are most susceptible to inhibition during the first 21 hours (proximal ridge stage). Beyond this, little effect is noted. Aeration of the regenerates during the first 20 hours improves regeneration in inhibitor water.

The supernatant from tissue breis of mature hydranths was collected by centrifugation (21,000 G). The minimum inhibitory dose was between 20 and 25 hydranth equivalents wherein stems required longer to regenerate (96 hours) and were reduced in size. Complete inhibition occurred between hydranth equivalents of 35 to 50 (a concentration of $\frac{1}{15}$ to $\frac{1}{10}$ in the culture medium). The tissue extract is highly resistant to sterilization, centrifugation and can be refrigerated indefinitely. The supernatant gives positive protein tests but freshly collected or boiled extract will readily dialyze and completely inhibit regeneration. Dialysis of the extract against running sea water for 24 hours with subsequent application to regeneration stems show a total loss of activity.

Attempts to breed an x-ray resistant clone of Paramecium. RALPH WICHTERMAN.[1]

In an attempt to breed radio-resistant paramecia by selection and cultivation of survivors of x-radiation, clonal cultures of *Paramecium multimicronucleatum* were irradiated repeatedly at intervals extending over a period of 13 months. For irradiation, 200 specimens were placed in each of 4 Nylon syringes (2 cc.) and, in one irradiation operation, given different dosages by the removal of a syringe at intervals from the x-ray generator. Most of the paramecia were irradiated with 100 to 250 kr in steps of 50 kr but occasionally higher dosages were used. Immediately after all irradiation exposures, survivors of the different dosages were placed in test-tubes of lettuce medium containing *Aerobacter aerogenes* as the food source. Upon regaining reproductive ability, progeny of survivors were then harvested, placed again in the syringes and irradiated as before. In some cases the next dosage was increased. As an example a clonal sample which received an earlier dosage of 100 kr later received 150 kr. After 13 successive and varied irradiation exposures of 4 sets of syringes, the clonal cultures of paramecia have received in this manner a cumulative dosage amounting to 1800 kr. Specimens are presently reproducing although at a slower rate of division than the unirradiated controls even after one year following the last irradiation. In addition to the reduced fission rate, the heavily irradiated clones after one year contain specimens which reveal the following: reduced swimming activity and altered behavior, reduced size, altered body shape and complete loss of all micronuclei. Unirradiated controls have three micronuclei.

Instead of being stream-line as in the controls, the paramecia from cumulatively irradiated clones are more ellipsoidal. Fission rate of well-fed controls occasionally reaches three divisions per day, generally not less than two. The successively irradiated clones that have received 1800 kr contain specimens which rarely reach two divisions per day even when last x-rayed one year earlier.

Using the same dosages, results indicate that specimens from the successively irradiated clones are more radio-sensitive than those irradiated for the first time.

[1] Part of a project aided by a contract between the Office of Naval Research, Department of the Navy, and Temple University (NR 135-263) and the Committee on Research, Temple University.

The micromanipulation of Arbacia eggs. F. J. WIERCINSKI.

Preliminary to a series of experiments with *Arbacia punctulata* egg cells it was necessary to develop a suitable microinjection technique. Egg and sperm suspensions were obtained from Arbacia by the electric method of stimulation. Fertilized and unfertilized eggs were pricked with a fine micropipette in a thin film of sea water suspended over a moist chamber. The micropipette was mounted in the upward position. With this technique many of the fertilized eggs did not develop to the two-cell stage because of injury from high surface tension phenomenon along the edge of the hanging droplet. A depression slide marked into two-millimeter squares containing 150 λ of sea water for 50 to 80 eggs was found to be adequate for normal development. After ten to fifteen minutes following fertilization, eggs were successfully microinjected with approximately 3 cubic micra per egg of 0.5% gelatin solution and showed normal development. The micropipette with a fine shaflet and a one-micron opening at the tip was mounted downward so that the injection was made into the cell as it rested on the surface of the slide. Dark field illumination with a magnification of 100 × was used to advantage to observe the position of the tip of the micropipette. The location of the eggs for control and experiment was noted by the squares on the slide. In late June, 1956, the fertilization percentage was 80. The jelly coat did not cling to the micropipette. In the middle of July, the jelly coat of the fertilized eggs was very tacky and the cells clung to the micropipette. The fertilization percentage was 95.

The physiology of the heart in marine fish. CHARLES G. WILBER.

For several years a variety of physiological studies have been made on the heart of marine fish available in the Woods Hole area. There have been suggestions in the literature that cardiac rate in fish may vary with size as in mammals. In order to test this, data from numerous species have been assembled. There is strong evidence that at a given temperature large fish have appreciably slower hearts than do smaller. For example here are a few average values in beats per minute for fish arranged in order of decreasing size: Roccus, 20; Opsanus, 40: Prionotus, 50; Tautogolabrus, 60; Fundulus, 100. These are values taken at an ambient temperature of 22° C. The toadfish, Opsanus, has a heart which is very refractory to many drugs: fairly large doses of decamethonium, atropine, and darstine cause no changes in the electrocardiogram of this species. Massive doses of darstine (80 mg. intravenously) cause an A-V dissociation and an eventual doubling of conduction time from pacemaker to ventricle. The latter chamber seems to be more sensitive to the drug than is the pacemaker. Decamethonium in the tautog results in impure auricular flutter; conduction time thru the ventricular muscle is unchanged but there is a significant prolongation of the refractory period. These studies are continuing.

A rapid method for recognition of specimens of Littorina littorea infected with trematode larvae. CHARLES H. WILLEY.

In response to the need for snails infected with *Cryptocotyle lingua* for experimental purposes an attempt was made to discover some external feature of the snail which would indicate that it was infected. The isolation method with examination of the water for emerged cercariae is too time-consuming and the snails show a high mortality rate following long isolation. Some snails even though infected will not shed cercariae during isolation for several days and the isolation method fails to identify immature stages of infection.

Observation indicated that the foot of an infected snail (*Littorina littorea*) becomes a dark yellow to brown in color in contrast to the whitish foot of uninfected specimens. To detect infected individuals, collections of snails are placed in sea water in tall glass containers such as battery jars and allowed to crawl up the sides. On the basis of the color of the foot seen from outside the glass, specimens can readily be sorted into infected and uninfected categories. In five collections of snails, the results have consistently been positive, checked by crushing and examining all the snails, both brown- and white-footed. Unless the specimens had a brownish foot, they were not infected. In one experiment 82 snails were assorted into two groups, 60 white foot and 22 with brown foot. Crushing and examination showed no infections among

those with white foot and 21 of the brown-footed specimens were infected. Similar results were obtained with other collections. Most of the infected snails harbored *Cryptocotyle lingua* but two other species were encountered in the infected group. Some were in early stages of infestation which would not have been detected by isolation techniques.

Induction of premature cleavage furrows in the eggs of Arbacia punctulata. ARTHUR M. ZIMMERMAN [1] AND DOUGLAS MARSLAND.[2]

These experiments indicate that the furrowing reaction, which normally is not scheduled to occur until telophase, can be induced to occur much earlier, starting, in fact, at about the 12th minute (at 20° C.) following insemination.

The induction treatment consists of pressure-centrifuging the fertilized eggs at high (8000–12,000 lbs./in.^{2}) pressure and at high (41,000 × G) force, for periods ranging up to 5 minutes. The temperature in all the experiments was kept constant at 20 ± 0.3° C.

The premature furrows appear 2–4 minutes subsequent to centrifugation, always at right angles to the centrifugal axis. Usually the furrows impinge from the equator of the cell, although sometimes they are displaced toward the centripetal end. Frequently they cut completely through the cell and do not recede. However, premature furrows, induced not more than 10 minutes prior to the normal time of furrowing, usually recede as soon as the normal furrows appear; and the normal furrows almost always come in at right angles to the premature ones. The eggs do not appear to be damaged appreciably, since the treated specimens gave rise to apparently normal plutei.

The greatest frequency of premature furrowing, which in many experiments involved virtually 100% of the eggs, was observed at 30–35 minutes after insemination. At 12,000 lbs./in.^{2}, a maximum frequency was obtained with 300 seconds of centrifugation, and there was a gradual decline in frequency with lesser durations of treatment. At lower pressures (8000 and 10,000 lbs./in.^{2}) the centrifugation times were longer.

Preliminary observations indicate that the induction of premature furrowing may be related to the rupturing of the nuclear membrane. No intact nuclei can be seen, either by phase or ordinary microscopy, in the pressure-centrifuged eggs, whereas nuclei can be seen in companion eggs centrifuged at the same force but not under pressure. Moreover, pressure-centrifuged premature furrowing eggs, stained by Feulgen (or the acetocarmine) technique, do not show intact nuclei, whereas the control cells do. The experimental eggs show just a small clump (sometimes two clumps) of densely packed Feulgen-positive material, lying in the vicinity of the furrow and displaying a diameter about ⅓ that of an intact nucleus. It is suggested, therefore, that the furrowing may be induced by a substance or substances released by the breaking of the nuclear membrane.

Pressure-centrifuge studies on mast cells. ARTHUR M. ZIMMERMAN,[1] JACQUES PADAWER [3] AND DOUGLAS MARSLAND.[2]

The pressure-centrifuge has been used extensively for studying sol-gel equilibria in Amoeba, Elodea and various marine eggs, but not in somatic mammalian cells. In this study, mast cells from rat peritoneal fluid were centrifuged under varying hydrostatic pressures at 41,000 × G and 20° C. It was found that the relative gel strength of these cells (expressed as the logarithm of the centrifugation time required to effect a distinct deformation in 25% of the cell population) is inversely proportional to the pressure. This relationship is similar to that found in all other cellular types that have been studied. Control mast cells, centrifuged at 41,000 × G at atmospheric pressure for periods up to 8 minutes (the longest centrifugation used in this

[1] Fellow of the Lalor Foundation, 1956.

[2] Work supported by the National Cancer Institute, Grant C-807 (cont.)

[3] Post-doctoral Research Fellow, American Heart Association. Supported in part by grants from the Damon Runyon Memorial Fund for Cancer Research (DRG 360) and from the American Heart Association.

study), were not measurably deformed. The deformation effected by the centrifugation was found to be spontaneously reversible at room temperature within approximately 30 minutes following return to atmospheric pressure.

Preliminary experiments show that mast cells obtained from 22-week-old rats may be appreciably more gelated than cells similarly obtained from 5-week-old animals. This result suggests that the aberrant morphology of mast cells encountered in old rats may be related to a progressively increasing gelational state of these cells during the aging process of the animal. Other cellular types of the peritoneal fluid (macrophagic elements and eosinophils) were not studied quantitatively. However, these cells were also deformed by the pressure-centrifugation. In fact, they seem to display a weaker gel structure than the mast elements.

LALOR FELLOWSHIP REPORTS

Sensory and motor relationships of a crustacean central ganglion. MELVIN J. COHEN.

Input-output relationships of the supraoesophageal ganglion in the lobster *Homarus americanus* were studied by stimulating statocyst afferents and recording the response evoked in occulomotor nerve fibers leaving the ganglion. Circulation to the exposed ganglion must be intact and the exposed portion of the central nervous system bathed in a balanced physiological solution in order for transmission through the ganglion to occur.

Movement of the statocyst sensory hairs evoked a response in occulomotor fibers which usually did not participate in the "spontaneous" activity of this nerve. Cutting both circumoesophageal connectives caused a burst of activity in the motor nerve followed by a gradual decrease in the number of spontaneously active units until only 1–3 fibers remained firing 15 minutes after cutting the connectives. The spontaneous activity in these remaining units was very rhythmic in contrast to the irregular spacing of impulses in the statocyst afferent neurons. Some occulomotor fibers increased in frequency up to 40/sec. when ipsilateral statocyst hairs were moved laterally toward the vertical, and decreased to 1–2 impulses/sec. when the same hairs were moved medially toward the horizontal. Here the frequency changes in the motor fibers seem to parallel those of the sensory neurons. Other occulomotor fibers increased in frequency only when statocyst hairs were moved medially toward the horizontal. This direction of hair movement is usually associated with a decrease in the frequency of firing in statocyst neurons. It appears, therefore, that a decrease in the level of spontaneous firing in certain statocyst afferents can serve as an adequate signal to the central nervous system and evoke a rise in frequency in specific fibers of the occulomotor nerve.

Invertebrate metabolism in vitro not affected by estradiol. DWAIN D. HAGERMAN.

Estradiol-17 β stimulates the oxidative metabolism of human endometrium and placenta *in vitro*. The stimulation can be demonstrated in tissue slices or cell-free soluble enzyme preparations, and is the result of a specific activation of a DPN-linked isocitric dehydrogenase. In a search for metabolic effects of estrogens in other species, a variety of invertebrate tissues were incubated in Warburg vessels in the presence or absence of estradiol (4×10^{-4} moles per liter). The incubation medium was filtered sea water, to which was added glucose (11.1 millimoles per liter) or potassium pyruvate (10 millimoles per liter) in some experiments. The gas phase was air. Tissues were incubated at 24–25° C. for four hours. Oxygen consumption was measured manometrically and conventional chemical techniques were used for the analysis of glucose, pyruvic acid, glycogen, and lactic acid.

No effect of estradiol on the rates of oxygen consumption, glycogen utilization, or lactate production was found in the ovaries and contained eggs of *Arbacia punctulata, Asterias forbesi, Mactra solidissima, Venus mercenaria, Busycon canaliculatum, Carcinides maenas, Homarus americanus,* or *Limulus polyphemus.* No effect of estradiol on glucose utilization or pyruvate utilization was found in the ovaries and contained eggs of *Arbacia punctulata, Asterias forbesi, Mactra solidissima,* or *Busycon canaliculatum.* Moreover, no effect of estradiol on these metabolic functions was found in the whole body of *Microciona prolifera,* the ctenidia or testes of

Loligo pealei, the gills or testes of *Callinectes sapidus*, the testes of *Homarus americanus*, or the liver of *Limulus polyphemus*.

Estrogens have been reported to be present in some of these tissues. Our metabolic experiments do not reveal any invertebrate estradiol-enzyme relationship similar to that found in man, and it is concluded that the estradiol-sensitive, DPN-linked isocitric dehydrogenase is not present in invertebrate tissues.

Methods for investigating the location of the photoperiodic receptors in insects. A. D. LEES.

Although it is well known that the induction of diapause in many insects and mites is controlled by the length of day, the site of absorption of the photoperiodic light energy has not yet been identified. In this connection the observation by Tanaka that the larvae of the oak silkworm *Antheraea pernyi* still respond to photoperiod after extirpation of the ocelli is of considerable interest. Two techniques which may prove of use in the identification of the receptors are being tested currently, using the larvae of *A. pernyi* as material. (I) Localized illumination can be achieved by the topical application of a transparent cellulose paint containing a blue-fluorescing substance, such as anthracene. The insects are then exposed for part of the photoperiod to a U.V. source with maximum emission at 365 mμ. The feasibility of this method rests upon the fact that insects in general exhibit greatest sensitivity to the blue region of the spectrum. (II) Using low incident light intensities to minimize light scattering, it may be possible to "silhouette" the sensitive areas by covering them with an opaque black paint. Since all five larval instars of *A. pernyi* are light-sensitive, the treated areas must be re-covered after each moult.

Contractility of glycerinated Vorticellae. LAURENCE LEVINE.

The technique of glycerination was applied to various species of Vorticellae in an attempt to elucidate some features of intracellular environment necessary for coiling of the spasmoneme (condensed myonemes).

Three species of Vorticellae, *campanula, nebulifera* and *convallaria*, maintained on an egg yolk infusion were used. Each species was very sensitive to glycerol because they invariably coiled tightly when immersed at 0° C. even in concentrations as low as 0.05 per cent. However, if the glycerol was 4 *mM* in EDTA (pH 7) such coiling was prevented. The glycerination procedure adopted, therefore, was immersion of animals previously washed in de-ionized water in 20 or 50% 0° C. glycerol and 4 *mM* in EDTA. Vorticellae glycerinated according to this recipe for as long as one month showed excellent preservation of the spasmoneme and other cellular features.

Physiological integrity of the spasmoneme was also retained. The coiling so characteristic of the living spasmoneme was produced by the addition of $CaCl_2$ in concentrations as low as 0.3 *mM* in 0.5 *M* KCl. Coiling was reversed through application of 4 *mM* EDTA and preparations were cycled repeatedly before contractility was lost. Contractility could not be restored through application of ATP and ions known to contract glycerinated rabbit psoas. Divalent ions such as magnesium and manganese also produced coils but were not as effective as calcium.

The retention of contractility was dependent upon species, concentration of glycerol and extraction time. Stalks of *Vorticella convallaria* lost contractility after short extraction in 20% glycerol, whereas coiling ability was maintained for longer periods in 50%.

These observations suggest that an extractable calcium-activated factor is responsible for spasmoneme coiling.

Regulation of arginine biosynthesis in Escherichia coli. W. K. MAAS.

Although *E. coli* cells are able to synthesize their amino acids from simple nitrogen and carbon sources such as ammonia and glucose, when an amino acid is supplied in the culture medium, the bacteria will utilize it in preference to synthesizing their own. In order to elucidate this regulatory mechanism, the effect of externally supplied arginine on its own biosynthesis

was studied. Arginine was chosen because most of the enzymes involved in its biosynthetic pathway have been extracted and the reactions catalyzed by them characterized. It was found that arginine inhibits the synthesis of transcarbamylase, the enzyme which couples ornithine with carbamyl phosphate to form citrulline. In growing cultures, in the presence of 10 micrograms/ml. of arginine, no transcarbamylase is formed. After removal of arginine the enzyme is resynthesized rapidly. Citrulline, on the other hand, does not inhibit synthesis of transcarbamylase. Studies are in progress on the effect of arginine on other enzymes involved in arginine biosynthesis.

The transcarbamylase system also offers an opportunity for studying the conditions required for the synthesis of a constitutive enzyme. Experiments have been carried out to see whether or not the presence of ornithine is necessary for enzyme synthesis. For these studies, a mutant unable to synthesize ornithine was used. The cells were first grown on arginine to exhaust transcarbamylase and then transferred to either citrulline or ornithine. It has found that transcarbamylase was resynthesized as rapidly in the presence of citrulline as in the presence of ornithine. These results indicate that, in contrast to the synthesis of adaptive enzymes, here the substrate may not function as an inducer for enzyme formation.

The ATPase activity of frog myosin. G. W. DE VILLAFRANCA.

In studying the ATPase activity of frog muscle it was found that methods normally used for preparation of myosin from rabbit muscle always resulted in actomyosin. Even centrifugation of purified actomyosin at 110,000 g in the presence of ATP failed to yield actin-free myosin. Myosin, however, was obtained by centrifuging twice precipitated actomyosin in 0.6 *M* KCl and 0.1 *M* $MgCl_2$ at 110,000 g for 30 minutes after the actomyosin had been previously cleared by centrifugation in 0.6 *M* KCl at that force for 20 minutes. The supernatant after Mg^{++} treatment was then treated with an equivalent amount of Versene to remove the ATPase inhibiting Mg^{++}. It was then precipitated twice by dilution with 10 volumes of cold, ion-free water and, between precipitations, dialyzed overnight against 3 changes of 0.6 *M* KCl. There was no change in viscosity of the myosin upon addition of ATP, it had an intrinsic viscosity approximately that of rabbit myosin, and it had pronounced ATPase activity (Q_P about 1200).

Three types of preparations (short extraction with Hasselbach-Schneider solution, 24-hour extraction with Weber-Edsall solution, and myosin prepared as described) showed the same ATPase characteristics and yielded Q_P-s ranging from 500–1400 at 24° C. The pH optimum was found to lie between pH 9 and 9.5 with occasional smaller peaks in the pH 6–7 region. Calcium activated strongly with an optimal concentration of 5×10^{-4} *M* while magnesium either inhibited or gave the same activity as the absence of divalent ions. Increasing the KCl concentration from 0.03 to 0.24 *M* progressively decreased activity. The enzyme split only 40–50% of the 10 min. P. Optimal activity was obtained at 24° C.; pre-incubation for 15 minutes resulted in slight inactivation (10%) at 37° C. and almost complete inactivation (94%) at 45° C.

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CONTENTS

Page

ANDERSON, JANE COLLIER

Relations between metabolism and morphogenesis during regeneration in Tubifex tubifex. II 179

BILEAU, SISTER M. CLAIRE OF THE SAVIOR

The uptake of I^{131} by the thyroid gland of turtles after treatment with thiourea 190

DETHIER, V. G., D. R. EVANS AND M. V. RHOADES

Some factors controlling the ingestion of carbohydrates by the blowfly 204

GIBOR, AARON

The culture of brine algae 223

GIBOR, AARON

Some ecological relationships between phyto- and zooplankton 230

GOLDSMITH, TIMOTHY H., AND DONALD R. GRIFFIN

Further observations of homing terns 235

PARK, HELEN D.

Modification of x-ray injury to Hydra littoralis by post-irradiation treatment with magnesium sulfate and glutathione 240

STUNKARD, HORACE W.

The morphology and life-history of the digenetic trematode, Azygia sebago Ward, 1910 248

TURNER, C. L.

Twinning and reproduction of twins in Pelmatohydra oligactis 269

TWAROG, B. M., AND K. D. ROEDER

Properties of the connective tissue sheath of the cockroach abdominal nerve cord 278

Abstracts of papers presented at the Marine Biological Laboratory:

Tuesday Evening Seminars 287

General Meetings 288

Lalor Fellowship Reports 318